Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus

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Acronyms and abbreviations

AE	Acridinium ester
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
BD	Becton, Dickinson and Company
bDNA	Branched chain DNA
BV	Bacterial vaginosis
C2CA	Circle-to-circle amplification
CCUG	Culture Collection University of Gothenburg (Gothenburg, Sweden)
CDC	Centers for Disease Control and Prevention (Atlanta, Georgia, United States of America)
CDS	Calibrated dichotomous sensitivity
CFU	Colony-forming units
CIA	Chemiluminescence assay
CIN	Cervical intraepithelial neoplasia
CLSI	Clinical and Laboratory Standards Institute
CMT	Cytosine DNA methyltransferase
CO2	Carbon dioxide
COSHH	Control of substances hazardous to health
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CTA	Cysteine trypticase agar
CV	Coefficient of variation
CVA	Cofactors-vitamins-amino acids
DALY	Disability-adjusted life year
DBS	Dried blood spot
DFA	Direct immunofluorescence assay
DGI	Disseminated gonococcal infection
DKA	Dual kinetic assay
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EIA	Enzyme immunoassay
EID	Early infant diagnosis
ELISA	Enzyme-linked immunosorbent assay
EQA	External quality assessment
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	United States of America Food and Drug Administration
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
FTA-Abs	Fluorescent treponemal antibody absorption
g	Acceleration due to gravity
GASP	Gonococcal Antimicrobial Susceptibility Surveillance Programme
GCMB	GC medium agar base
gG	Glycoprotein G
GKNP	Glucose-potassium-sodium-phosphate solution
GUD	Genital ulcer disease
HBV	Hepatitis B virus
HC/HC2	Hybrid Capture/Hybrid Capture 2
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HIVDR	HIV drug resistance
HPA	Hybridization protection assay
HPV	Human papillomavirus
HR	High-risk types
HSIL	High-grade intraepithelial lesion
HSV	Herpes simplex virus
ICT	Immunochromatographic test
IF	Immunofluorescence
IFA	Immunofluorescence assay
IgA/IgG/IgM	Immunoglobulin A, G, M
IMDM-VGA	Iscove's modified Dulbecco medium
IP	Immunoperoxidase
IQA	Internal quality assessment
IQC	Internal quality control
ISO	International Organization for Standardization
KOH	Potassium hydroxide
LDA	Laboratory-developed assays
LGV	Lymphogranuloma venereum
LIA	Line immunoassay
LJ	Levey–Jennings plots
LPS	Lipopolysaccharide
LR	Low-risk types
LSIL	Low-grade intraepithelial lesion
MALDI-TOF	Matrix-assisted laser desorption ionization–time of flight
MH	Mueller–Hinton
MIC	Minimal inhibitory concentration

MIF	Microimmunofluorescence		RNA	Ribonucleic acid
MOMP	Major outer membrane protein		RPR	Rapid plasma reagin
MS	Mass spectrometry		RRP	Recurrent respiratory papillomatosis
MSM	Men who have sex with men		RT	Reverse transcriptase
MTM	Modified Thayer–Martin medium		SARA	Sexually acquired reactive arthritis
NAAT	Nucleic acid amplification test		SD	Standard deviations
NAH	Non-amplified nucleic acid hybridization assays		SDA	Strand displacement amplification
			SNP	Single nucleotide polymorphism
NASBA	Nucleic acid sequence-based amplification		SOP	Standard operating procedure
NCNGU	Non-chlamydial NGU		SPG	Sucrose-phosphate-glutamate
NCTC	National Collection of Type Cultures		spp.	Species
NGU	Non-gonococcal urethritis		STI	Sexually transmitted infection
NIBSC	National Institute for Biological Standards and Control		TC	Target capture
			TMA	Transcription-mediated amplification
NPV	Negative predictive value		TOC	Test-of-cure
nvCT	New variant of *Chlamydia trachomatis*		TPHA	*Treponema pallidum* haemagglutination assay
NYC	New York City agar		TPPA	*Treponema pallidum* passive particle agglutination assay
OF	Oral fluid			
Pap	Papanicolaou		TRUST	Toluidine red unheated serum test
PBS	Phosphate-buffered saline solution		TSB	Trypticase soy broth
PCR	Polymerase chain reaction		UK NEQAS	United Kingdom National External Quality Assessment Service
PET	Preformed Enzyme Test			
PID	Pelvic inflammatory disease		UNAIDS	Joint United Nations Programme on HIV/AIDS
PIP	Prolyliminopeptidase			
PMNL	Polymorphonuclear leukocytes		VDRL	Venereal Disease Research Laboratory
POC	Point-of-care		VL	Viral load
PPV	Positive predictive value		VVC	Vulvovaginal candidiasis
PR	Protease regions		WB	Western blot
QA	Quality assurance		WHO	World Health Organization
QC	Quality control		WHO/TDR	WHO Special Programme for Research and Training in Tropical Diseases
QMS	Quality management system			
RCA	Rolling circle amplification		WR	Wasserman reaction
RCUT	Rapid carbohydrate utilization test			
RHR	Department of Reproductive Health and Research			

Preface

Sexually transmitted infections (STIs), including those caused by the human immunodeficiency virus (HIV) types 1 and 2, remain an important focus area for global public health. This is due to the high morbidity associated with STIs, such as the sequelae of reproductive tract infections, cervical cancer, congenital syphilis, ectopic pregnancy and infertility, as well as the morbidity of HIV-related illness and death from acquired immunodeficiency syndrome (AIDS). Public health strategies for STI control include promotion of safer sexual behaviour and provision of condoms (primary prevention), as well as early and efficacious management of patients with STIs, using either syndromic or etiological management approaches.

With the syndromic management approach, accessible, affordable, and effective management of individuals with STIs relies on utilization of flowcharts (algorithms) for each STI syndrome. The flowcharts enable diagnoses of common STI syndromes, provision of current country-specific appropriate treatments, advice on the management of sexual partners, and emphasis on the importance of same-visit HIV testing. The flowcharts should be based on local etiological and antimicrobial susceptibility data provided through periodic laboratory-based surveys. In general, laboratory tests are not undertaken for most STI patients who receive syndromic management. However, it may be appropriate to take specimens for laboratory testing from those patients failing first-line therapies, to establish diagnoses and/or to determine if treatment failure was due to antimicrobial resistance. In the countries that can afford the etiological diagnostic approach, the laboratory plays a much greater role in terms of diagnosis of specific STI pathogens and determination of antimicrobial susceptibility. Laboratories also have a key role to play in terms of STI surveillance and research programmes within both resource-poor and more wealthy nations.

The World Health Organization published an earlier version of this manual, entitled *Laboratory diagnosis of sexually transmitted diseases*, in 1999, with the objective of providing a comprehensive guide of standard procedures for isolating, detecting, and diagnosing STIs for microbiologists and medical technologists. It was conceived as a practical bench manual, tuned to the needs and capacities of laboratories at different levels in the health-care system. The handbook subsequently proved very popular within both countries and individual laboratories.

Since publication of the 1999 manual, there have been a number of key advances in diagnostic procedures, in particular, with respect to nucleic acid amplification and rapid point-of-care tests, as well as antimicrobial susceptibility testing methodologies and recommendations. A number of international experts have extensively updated chapters from the 1999 manual accordingly. In addition, this revised version includes new chapters on a number of topics, including diagnostic techniques for *Mycoplasma genitalium*, point-of-care tests for STIs, and laboratory quality management. While this updated manual does cover the most important STI pathogens, it should not be viewed as comprehensive, and the reader may need to consult other reference resources for more information, for example, with respect to national STI policies, antimicrobial susceptibility testing guidelines, medico-legal issues, and STI testing in minors.

This new manual, *Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus*, provides a basic understanding of the principles of laboratory tests in the context of screening and diagnostic approaches, as well as antimicrobial susceptibility testing, as components of STI control. As with the 1999 manual, this manual covers each disease in a separate chapter that provides detailed information on specimen collection, transport, and laboratory testing. Two useful annexes covering equipment, tests, media, reagents, and stains are included at the end of the manual.

It is envisaged that this updated manual will be informative to administrators, programme managers, medical staff and nurses, as well as to the primary target audience, which remains microbiologists and medical technologists. The manual may be a useful tool to assist in the procurement of the most appropriate diagnostic tests for individual settings, ideally through national and/or local expert advisory committees. The manual also is a valuable resource for those

training students, and for the students themselves, both within and outside of the laboratory environment. Finally, it is anticipated that the growth in diagnostic products and methodologies will continue in the next few years; therefore, it will be important for all readers to keep themselves up to date with the latest developments in the field.

Acknowledgements

Chapter 1

Choosing tests for sexually transmitted infections

1.1 Introduction

More than 30 bacterial, viral, and parasitic pathogens are transmissible sexually and constitute a group of infections referred to as sexually transmitted infections (STIs). Although some of the pathogens can be acquired through routes other than sexual transmission, epidemiologically, sexual contact is more important for their transmission from one person to another (Table 1.1).

Laboratory and point-of-care (POC) tests are potentially powerful contributors to the management and control of STIs through facilitation of prevention of STI transmission and their sequelae. The large numbers of STIs as well as the variety of potential tests for each STI make the appropriate choice of diagnostic tests difficult. At the present time, a wide variety of available STI tests have attributes and potential limitations that could affect how they might be used to enhance STI control. Further, in an era of limited resources, decision-making about which and how many STIs to invest in for testing, who to test, and which of the multiple available tests to use for a designated purpose can be difficult. Test selection should reflect a prioritization process that considers infection prevalence, the impact of the infections and their complications on individuals and populations, test performance characteristics, the cost of the tests, and the reasons that testing is being performed.

Tests for STIs may be used for a variety of different purposes that, in turn, may affect the choice of tests. These different reasons for testing include for purposes of surveillance, for validation of syndromic management algorithms, for quality assurance (QA), for diagnosis of persons with signs and symptoms of possible STI, for screening of asymptomatic at-risk persons, and for antimicrobial susceptibility testing. Some key elements of these terms are outlined as follows:

- **Surveillance.** Surveillance is the systematic collection, collation, and analysis of data to determine how common an infection is within a community or population. It is an essential element of planning for

STI control efforts. Investment in surveillance for uncommon infections or infections with little direct impact on public health may not be highly prioritized if resources are limited, if the infection is uncommon (e.g. chancroid, particularly in developed nations), or if the associated morbidity is modest (e.g. pubic lice). Generally, the time needed to obtain a test result for surveillance purposes is not critical. In some instances, specimens collected for surveillance may also be used to monitor other clinically important factors such as antimicrobial resistance (e.g. in *Neisseria gonorrhoeae*).

- **Validation of syndromic management.** Syndromic diagnosis is a valuable element of STI control efforts, providing a rapid diagnostic assessment that can then be used to guide timely therapy for persons with signs and symptoms of infection. In settings where syndromic diagnosis represents an element of STI management efforts, periodic laboratory testing of patients diagnosed and treated using syndromic management algorithms for STIs should be performed to ensure that syndromic diagnosis is succeeding in identifying the infections targeted for intervention. In situations in which syndromic diagnosis is not resulting in treatment of its targeted STI, efforts to evaluate the reasons for failure are warranted. Information gained through periodic use of testing to evaluate the quality of clinical services can be used for feedback and steps to improve patient care.

- **Quality assurance/improvement.** The large area of QA includes both quality control performed to assure that tests are performing as designed, and quality assessment performed to ensure that tests are being used appropriately and that all steps needed to use tests to manage the patients being tested are being carried out correctly. STI test accuracy may be impacted by a variety of factors, including variation in reagents, equipment function,

Table 1.1: Main sexually transmitted pathogens and the diseases they cause

Pathogen	Clinical manifestations and other associated diseases
Bacterial infections	
Neisseria gonorrhoeae	GONORRHOEA **Men:** urethral discharge (urethritis), epididymitis, orchitis, infertility **Women:** cervicitis, endometritis, salpingitis, pelvic inflammatory disease, infertility, preterm rupture of membranes, perihepatitis; commonly asymptomatic
Chlamydia trachomatis	CHLAMYDIAL INFECTION **Men:** urethral discharge (urethritis), epididymitis, orchitis, infertility **Women:** cervicitis, endometritis, salpingitis, pelvic inflammatory disease, infertility, preterm rupture of membranes, perihepatitis; commonly asymptomatic **Both sexes:** proctitis, pharyngitis, Reiter's syndrome **Neonates:** conjunctivitis, pneumonia
Chlamydia trachomatis (serovars L1–L3)	LYMPHOGRANULOMA VENEREUM **Both sexes:** ulcer, inguinal swelling (bubo), proctitis
Treponema pallidum	SYPHILIS **Both sexes:** primary ulcer (chancre) with local adenopathy, skin rashes, condylomata lata; bone, cardiovascular, and neurological damage **Women:** pregnancy wastage (abortion, stillbirth), premature delivery **Neonates:** stillbirth, congenital syphilis
Haemophilus ducreyi	CHANCROID **Both sexes:** painful genital ulcers; may be accompanied by bubo
Klebsiella (Calymmatobacterium) granulomatis	DONOVANOSIS (GRANULOMA INGUINALE) **Both sexes:** nodular swellings and ulcerative lesions of the inguinal and anogenital areas **Men:** urethral discharge (nongonococcal urethritis) **Women:** cervicitis, endometritis, probably pelvic inflammatory disease
Mycoplasma genitalium	**Men:** urethral discharge (nongonococcal urethritis) **Women:** cervicitis, endometritis, probably pelvic inflammatory disease
Viral infections	
Human immunodeficiency virus (HIV)	ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) **Both sexes:** HIV-related disease, AIDS
Herpes simplex virus type 2 Herpes simplex virus type 1 (less common)	GENITAL HERPES **Both sexes:** anogenital vesicular lesions and ulcerations **Neonates:** neonatal herpes (often fatal)
Human papillomavirus	GENITAL WARTS **Men:** penile and anal warts; carcinoma of the penis **Women:** vulval, anal, and cervical warts, cervical carcinoma, vulval carcinoma, anal carcinoma **Neonates:** laryngeal papilloma

Table 1.1: Main sexually transmitted pathogens and the diseases they cause (*continued*)

Pathogen	Clinical manifestations and other associated diseases
Viral infections (*continued***)**	
Hepatitis B virus	VIRAL HEPATITIS **Both sexes:** acute hepatitis, liver cirrhosis, liver cancer
Cytomegalovirus	CYTOMEGALOVIRUS INFECTION **Both sexes:** subclinical or nonspecific fever, diffuse lymph node swelling, liver disease, etc.
Molluscum contagiosum virus	MOLLUSCUM CONTAGIOSUM **Both sexes:** genital or generalized umbilicated, firm skin nodules
Kaposi sarcoma associated herpesvirus (human herpesvirus type 8)	KAPOSI SARCOMA **Both sexes:** aggressive type of cancer in immunosuppressed persons
Protozoal infections	
Trichomonas vaginalis	TRICHOMONIASIS **Men:** urethral discharge (nongonococcal urethritis); often asymptomatic **Women:** vaginosis with profuse, frothy vaginal discharge; preterm birth, low-birth-weight babies **Neonates:** low birth weight
Fungal infections	
Candida albicans	CANDIDIASIS **Men:** superficial infection of the glans penis **Women:** vulvo-vaginitis with thick curd-like vaginal discharge, vulval itching, or burning
Parasitic infestations	
Phthirus pubis *Sarcoptes scabiei*	PUBIC LICE INFESTATION SCABIES

and technical proficiency. As a result, periodic testing of standardized specimens to assess laboratory proficiency and to assure the expected test accuracy is recommended. Standardized specimens may be obtained from accrediting organizations or generated within the laboratory. Evaluation of standardized specimens may identify a need for re-training or evaluation of the quality of individual components of the laboratory test.

- **Diagnosis.** Symptoms of common STIs tend to be non-specific and typically have a variety of different potential causal agents that may require different treatments. As a result, diagnostic testing is helpful both for purposes of accurate diagnosis and for guiding the management of sexual partners as well as for QA of syndromic management algorithms, as noted above. When used for diagnosis, the time required for test results to become available to guide management should be considered in choosing tests, since infected persons may transmit infection to others, may suffer complications of infection, or may be lost to follow-up in the interval between testing and notification of test results (*1, 2*). When diagnostic testing is performed as part of clinical services, it is sometimes useful to monitor the interval between testing and treatment of persons with positive tests as a quality-of-care measure.

- **Screening.** Screening is an essential element of optimal STI management and control strategies that builds on the contributions of syndromic management and diagnostic testing. All STIs may occur in an asymptomatic form or be unrecognized by infected persons. Despite the absence of identified symptoms, persons with asymptomatic infections may be at risk for transmission to others and for complications of infection. As a result, screening (i.e. testing of at-risk persons without recognized signs or symptoms) will identify infected persons, thus reducing their risks for complications or for transmission of infections. As with testing for diagnosis, the time interval between testing and provision of treatment, as well as the proportion of persons receiving treatment, if some are lost to follow-up, are useful quality measures. Screening for STIs may be more cost-efficient if it can be targeted to at-risk population subgroups; targeting is often best accomplished using surveillance data.

- **Determination of antimicrobial susceptibility.** For some STIs (*N. gonorrhoeae* is a noteworthy example), the continuing development of resistance has periodically led to changes in recommended therapy. Systematic surveillance of antimicrobial susceptibility and/or testing of specific isolates for susceptibility to the antimicrobial agents used for therapy provide information that can be used to adjust treatment recommendations in anticipatory fashion, optimally before treatment failure becomes a problem. Determination of antimicrobial susceptibility is best performed on living clinical isolates of cultured organisms and is typically better used to guide recommended therapy for populations rather than for individual patient management. For the most part, surveillance for antimicrobial resistance is best conducted by reference laboratories using specimens collected from a spectrum of geographically representative sites and populations of interest.

1.2 Types of diagnostic tests

Scientific progress has provided a broad array of tests for identification of STIs. These tests vary greatly in terms of their level of complexity (i.e. the technical requirements for optimal test performance), in the costs required to perform them (both material- and labour-related), and

in terms of performance. Thus, each type of diagnostic test has its own strengths and shortcomings. As a result, in some settings, the most accurate test may not be the best test for use if it is so expensive that it cannot be utilized to test large numbers of at-risk persons or if the test is so complex that results are not available in a timely fashion to guide patient management. Finally, in some instances, laboratory tests for STIs are available in a variety of formats and platforms, which influences the number of tests that can be performed in a given time period. Thus, test throughput (the numbers of tests completed in a given period of time) is also a consideration in test selection. In some settings, higher or lower volumes of tests will make some tests or test platforms preferable.

In general, diagnostic tests can be separated into at least three different types. First, direct detection of microorganisms themselves is the most obvious approach to STI diagnosis. This may be accomplished through the use of microscopy and appropriate staining or wet preparation to visualize pathogens. Culture, antigen detection, or nucleic acid detection using either amplified or non-amplified nucleic acid detection tests are often more sensitive than microscopy but may have more complex technical requirements for optimal test performance and may increase the interval between testing and the availability of test results (rapid POC tests help to overcome the latter potential limitation). Each of these approaches has its own strengths and weaknesses. Microscopy, particularly when performed while patients are present, may provide immediate results to guide management decisions but, like other tests, requires specialized equipment (the microscope), may require electrical power or special stain procedures, and is dependent on the training and experience of the microscopist.

In contrast, other laboratory-based tests, such as culture or nucleic acid amplification testing, may require special methods of specimen transport and specialized equipment and procedures for optimal performance, thus delaying the availability of results for immediate management decisions.

Secondly, for many important STIs (syphilis and HIV represent common examples), detection of the host

response to infection (antibodies) represents a favoured diagnostic test. The strength of serological tests is that they may be useful not only for purposes of diagnosis but also for surveillance. All serological tests have occasional false-positive test results. The problem of false-positive serological tests can often be reduced by testing specimens found to be positive on an initial, screening serological test using a second, confirmatory serologic test, which targets a different antigen (use of confirmatory tests is discussed in more detail in the sections on syphilis and HIV serological testing). Some serological tests may be able to differentiate recently acquired infections from more longstanding or previously treated infections through detection of immunoglobulin M (IgM) for recent infections. A shortcoming of serological diagnosis is that antibodies to an STI pathogen may persist long after successful treatment. As such, serological testing of populations may be an indication of total cumulative infection rather than more recently acquired infections.

Thirdly, there are tests that detect microbial metabolites, such as materials altering the pH of genital secretions and biogenic amines. These tests are useful adjuncts for diagnostic purposes in some settings. An example of this is the importance of pH and whiff/amine tests in the diagnosis of bacterial vaginosis.

1.3 Test performance

Ultimately, the value of tests for STI detection also depends a great deal on their performance (Table 1.2). As measures of performance, calculations of sensitivity and specificity (3)—provided such calculations are performed using sufficiently large sample size—represent reliable estimates of the overall performance of tests. However, the predictive values (both positive and negative) of such tests can vary substantially from population to population depending on the prevalence of an infection in the community.

Thus, tests that have substantial false-positive test rates and test for relatively uncommon (i.e. low prevalence)

Table 1.2: Effect of sensitivity, specificity, and prevalence, using primary test only or supplementary test additionally, on positive predictive value

Population prevalence = 1%					
	A	B	C	D	E
Primary test sensitivity/specificity	99%/99%	99%/99.9%	99%/99%	99.5%/99.5%	99.5%/99.5%
Supplementary/confirmatory test sensitivity/specificity	ND	ND	99%/99%	ND	99.5%/99.5%
Number tested	1000	1000	11	1000	15
Negatives					
Total number	980	989	1	985	5
True negatives	980	989	1	985	5
False negatives	0	0	0	0	0
Positives					
Total	20	11	10	15	10
True positives	10	10	10	10	10
False positives	10	1	0	5	0
Positive predictive value (PPV)	50%	91%	100%	67%	100%

ND, not determined.

infections may have a low positive predictive value despite having high sensitivity for detection of infection. In general, published peer-reviewed literature provides reliable estimates of test sensitivity and specificity. However, for any specific setting, local disease prevalence will partially determine the predictive value of laboratory tests. Thus, surveillance to determine local disease prevalence provides important data for choosing tests for STIs.

1.4 Laboratory types and roles

Although the traditional method for the diagnosis of STIs has been through laboratory analyses to determine the etiological agents, not all tests need to be performed in all laboratories for all purposes. Laboratory-based diagnosis of STIs tends to be expensive in terms of equipment, reagents, infrastructure, and maintenance. Even more importantly, particularly in resource-constrained settings, the levels where most patients with STIs are processed have no laboratory facilities (*2*). To accommodate for such a situation where laboratory facilities cannot be made available, the World Health Organization developed guides on the use of the syndromic approach for the management of some STIs and advocated its use since the mid 1980s after field evaluations of its performance (*3*). Subsequently, a number of field studies showed that the syndromic approach performs well in the management of men with symptomatic urethral discharge and in the management of men and women with bacterial genital ulcer disease (*4*, *5*). This is not the case, however, for the diagnosis and management of cervical infection with *N. gonorrhoeae* or *C. trachomatis*, except in cases in which examination shows cervical mucopus, cervical erosions, and cervical friability, as well as a history of bleeding between menses and during sexual intercourse (*6*). Nonetheless, syndromic diagnosis and treatment has the advantages of providing immediate care at the patient's first port of call for assessment and it is inexpensive—the patient, the service provider, or the state incurs no direct laboratory costs. Using the syndromic approach also enables standardization of diagnosis, treatment, and reporting in a particular setting or situation. Patients with suspected STIs can be managed at outlets such as primary health-care facilities, family planning clinics, and private practitioner

consulting rooms as well as in specialized STI clinics themselves (*7*). Inevitably, the syndromic approach has the potential to overdiagnose and overtreat patients who may not be infected with any or some of the presumed causative organisms for the syndrome in question (*8*).

Therefore, to support the syndromic approach to diagnosis, local clinical laboratories should be encouraged to perform the tests needed to facilitate clinical management of persons with and at risk for STI. In some settings where specimen transport is not problematic, economies of scale make specimen processing in central laboratories as timely as, and more efficient than, efforts to perform testing at local sites. All laboratories need not perform reference laboratory testing activities such as antimicrobial susceptibility testing.

The laboratory systems can be arbitrarily categorized into three levels based on the levels of care and treatment services supported by each category. However, it should be borne in mind that laboratory infrastructure and diagnostic capabilities vary widely between the resource-constrained and industrialized countries. Consider the following categories as a general guide:

1. Peripheral laboratories supporting the primary health-care service level have limited laboratory equipment and minimally trained laboratory staff. These laboratories are designed to provide rapid on-site diagnosis of STIs.

2. Intermediate-level laboratories supporting the primary health-care service laboratories and intermediate/district level clinics and hospitals.

3. Central-level laboratories supporting the tertiary health-care clinics, including specialized reference STI clinics as well as lower level health-care clinic laboratories and clinics.

Although rapid POC tests may lack sensitivity, generally they have good specificity characteristics and may offer savings in the management of some conditions such as vaginal discharge at the peripheral health-care level.

This manual describes testing approaches ranging from basic, inexpensive approaches to sophisticated,

expensive approaches. Programme managers and laboratory experts, in collaboration with policy-makers, should determine the feasibility and utility of incorporating tests at the different levels of health-care facilities. The choices will also depend on whether different levels of the health-care facilities use the syndromic approach (basic or modified), the etiologic approach, or both of these approaches.

1.5 Putting the pieces together

There is no single optimal test for the detection of agents causing STIs. For programmatic decision-making, the multiple STIs and impacted populations must be considered as a matrix to guide not only which laboratory test is most appropriate for the community under consideration, but also what proportion of the total available resources (budget, personnel, etc.) should be allocated for each STI to be tested for (Table 1.3).

Decisions regarding the choice of tests must be conducted in the context of the prevalence of the infection under consideration, the impact of the infection

on the community, resources available to support testing and treatment, and the prioritization of that infection within the context of other STIs. Further, in choosing diagnostic tests, factors such as complexity, time to test results, and cost are also essential considerations. Mathematical modelling has clearly demonstrated that, in situations and settings in which test results are made available at the time of initial patient evaluation and where patients may be delayed in receiving treatment if they do not receive treatment at the time of evaluation, less sensitive tests provided at point of care may actually increase the numbers of persons treated and reduce complications of infection when compared to more sensitive tests that require increasing time to get test results (*2*).

1.6 References

1. Geisler WM et al. The natural history of untreated *Chlamydia trachomatis* infection in the interval between screening and returning for treatment. *Sexually Transmitted Diseases*, 2008, 35(2):119–123.

2. Gift TL et al. The rapid test paradox: when fewer cases detected lead to more cases treated: a decision analysis of tests for *Chlamydia trachomatis. Sexually Transmitted Diseases*, 1999, 26(4):232–240.

3. Kuypers J, Gaydos CA, Peeling RW. Principles of laboratory diagnosis of STIs. In: Holmes KK et al., eds. *Sexually transmitted diseases*, 4th ed. New York, McGraw-Hill Medical, 2008:937–958.

4. Adler MW et al. Sexual health and care: sexually transmitted infections: guidelines for prevention and treatment. [Occasional paper]. London, ODA, 1996.

5. *Management of patients with sexually transmitted diseases*. Geneva, World Health Organization, 1991 (WHO Technical Report Series No. 810).

6. Alary M et al. Evaluation of clinical algorithms for the diagnosis of gonococcal and chlamydial infections among men with urethral discharge or dysuria and women with vaginal discharge in Benin. *Sexually Transmitted Infections*, 1998, 74(Suppl. 1):S44–49.

7. Moherdaui F et al. Validation of national algorithms for the diagnosis of sexually transmitted diseases in Brazil: results from a multicentre study. *Sexually Transmitted Infections*, 1998, 74(Suppl. 1):S38–43.

8. Vuylsteke B. Current status of syndromic management of sexually transmitted infections in developing countries. *Sexually Transmitted Infections*, 2004, 80(5):333–334.

Table 1.3: Potential factors influencing choice of tests for STIs

1. Purpose of testing
• Surveillance
• Quality assurance
• Evaluation of syndromic diagnosis
• Diagnosis
• Screening
• Antimicrobial susceptibility testing
2. Test-specific considerations
• Performance (sensitivity, specificity, predictive value)
• Specimen collection and transportation requirements
• Prevalence
• Associated morbidity
• Resources
• Financial
• Personnel
• Infrastructure (utilities, etc.)
• Relative importance among other priorities

Chapter 2

Quality management in the laboratory

2.1 Introduction

The purpose of a quality system is to ensure that all reports that are used for patient management produced by the laboratory are of high quality. To maintain a high quality of service, laboratories should strive to have a quality improvement programme and to become accredited to a suitable national or international body, such as the International Organization of Standardization (ISO; www.iso.org). Accreditation involves an external audit of the ability to provide a service of high quality by declaring a defined standard of practice, which is confirmed by peer review. However, it does not include assessment of the appropriateness of the test chosen for the diagnosis of the particular infection or for the population to be tested (see Chapter 1).

To have a common shared understanding, Table 2.1 defines the terms used in this chapter.

Accreditation is a time-consuming and expensive procedure and not attainable by many laboratories, but all laboratories should work towards improving any procedure that affects the accuracy of information that guides patient management. In many parts of the world, systems have been developed to strengthen laboratory systems by implementing improvement in a stepwise manner against a checklist (1–5).

Table 2.1: Definitions of key terms used in the quality management chapter

Accreditation	External audit of laboratory procedures that contributes to the quality of the report.
Quality management system (QMS)	Framework for taking a systematic approach to managing the quality of the laboratory procedures.
Quality assurance (QA)	A systematic process to ensure fulfillment of quality requirements for a product or service.
Standard operating procedure (SOP)	A document with a detailed description of the individual procedure for each protocol or test used within the laboratory. The purpose of an SOP is to ensure the operations are performed correctly and always in the same manner. An SOP should be available at all times in the laboratory.
Internal quality assessment (IQA)	Performed by the periodical resubmitting and testing of randomly selected anonymised clinical samples within the same laboratory to ensure reproducibility of the results.
External quality assessment (EQA)	Allows the introduction of samples of known but undisclosed content into a laboratory's routine testing procedure from an independent source.
Internal quality control (IQC)	Used to detect problems or failure in one or more reagents with a test.
Evaluation of tests	A systematic and extensive process that compares different systems designed to perform the same or similar functions.
Validation of tests	Used to examine the whole process that is being used to check that results are correct.
COSHH	Control of substances hazardous to health.

2.2 Quality management

The quality system should be described in a quality manual that describes the management system for the laboratory. This serves to inform the laboratory's own management and staff and provide information for customers of the laboratory services and for any accreditation body.

Organization and management

An organizational structure is necessary for the laboratory where the roles and responsibilities of each individual are clear. This will usually be a hierarchical structure headed by a director or head of department, with section or unit heads, a laboratory manager, and range of health-care scientists of different grades. One individual should take the lead for quality; this could be either a dedicated role or an integral part of a wider job description. The quality manual should include an organogram of the roles of individuals and their line management and a description of their responsibilities. Each staff member should have a detailed job description, which should be reviewed annually.

Staff training and registration

All staff within the laboratory should be suitably trained and become registered, where possible, to a national body. Staff should receive training in all methods they will perform in their daily work and should be checked for competency on a regular basis. A training record should be kept for each staff member. They should also participate in ongoing training to maintain their expertise and competency. Auditing of procedures in the laboratory should be part of a good quality system and will be required for accreditation. Individual responsibilities should be supported by the following meetings of a series of groups within the laboratory:

- *Management or senior staff meetings* should be attended by the director or head of the laboratory, the laboratory manager, section heads, QA manager, laboratory safety officer and director's personal assistant. This group should meet regularly—typically six to eight times per year—to discuss relevant matters, including finance, administration, staffing, equipment, IQA and EQA, accreditation, and any other raised matters affecting the delivery of the laboratory

service. Minutes with action points should be kept of these meetings and reviewed at subsequent meetings.

- *All-staff meetings* should be attended by all grades of laboratory staff. This group should meet at least eight times a year to discuss relevant matters, including finance, administration, staffing, safety, accreditation, and any other raised matters. Minutes with action points should be kept of these meetings and reviewed at subsequent meetings.

- *Safety management meetings* should be attended by the director or head of the laboratory, laboratory manager, laboratory safety officer, deputy safety officer, section heads, a representative of staff and the health and safety adviser (if available). This group should meet at six-monthly intervals or as appropriate. Minutes should be posted on the laboratory notice board and freely available.

- *Annual management meetings* should be held and reports prepared to review the complete performance of the laboratory and the service it provides to its customers.

Standard operating procedures

All procedures used within the laboratory should be documented as SOPs and are critical in reducing errors in variation in testing. The principle details of the reagents and methodology, including internal controls and interpretative criteria, should be included. SOPs should be written by individuals performing the method/test and authorized by a senior member of staff. SOPs should cross-reference to risk assessments and safety information (chemical and biological COSHH) and should be reviewed and updated. No procedure should be undertaken in the laboratory without SOPs being in place and readily accessible in the laboratory for daily use. SOPs should be reviewed regularly, checked and authorized by senior technical staff as part of the laboratory QMS. In some countries, local SOPs can be informed by national SOPs.

Evaluation and validation of tests is essential in any laboratory to provide an evidence-based assessment of the performance capabilities of a test before it is incorporated into the service offered.

- **Evaluation** is a systematic and extensive process that compares different systems designed to perform the same or similar functions. Examples of evaluations within microbiology include comparison of different methods designed to detect the same marker/target, comparison of different culture media to isolate the same organism, or comparison of different equipment with the same function. Evaluation findings should be fed back to interested parties, e.g. via publication of results. Where two kits have equivalent performance characteristics, the one that is easier to use, cheaper, faster, or requires a more easily obtainable sample might be preferred.

- **Validation** is used to examine the whole process that is being used to check that results are correct. Each laboratory should validate their ability to achieve acceptable results with the method or system in question. To document this ability, each laboratory should produce a validation file for each method or system. The file should include a range of information and have a different emphasis depending on whether the laboratory is using a commercial system or has developed a system in-house. Typically, the file would include sections such as evaluation data, tests on known samples, workbooks, relevant publications, ongoing quality control data, relevant SOPs, error logs, and customer complaints.

The intention of validation is to provide documentary evidence that a diagnostic test or piece of equipment is performing within manufacturer's specifications. This may involve results of experiments to determine its accuracy, sensitivity, reliability, and reproducibility. A validation may be extensive (for example, to validate a newly developed in-house method) or narrow in scope (for example, to validate a commercial method) that is already in use and has had minor modifications.

For methods already in use for which no specific existing validation is in place, it is important to provide documentary evidence that supports reasons for their use. It is usually sufficient to prepare a file based on historical evidence, such as results from comparisons or other studies undertaken, copies of published papers, EQA, IQA, and IQC results, etc. Work book records can be cross referenced if appropriate in the validation report.

Quality assurance (QA)

QA is fundamental to the work of the laboratory, to maintain the quality of the service and ensure that results are both accurate and reproducible, where accuracy is the closeness of agreement between the mean value obtained from a large series of test results and an accepted reference value and reproducibility is the ability to produce essentially the same diagnostic result irrespective of variations in operator, test batch, laboratory, or validated ancillary equipment.

There are two main QA elements: quality assessment, both IQA and EQA; and quality control (QC), which encompasses evaluation and validation of tests, IQC, and equipment evaluation and monitoring (Fig. 2.1).

Quality control (QC)

IQC is used to detect problems or failure in one or more reagents with a test. For instance, for culture systems, one or more known control strains can be used to ensure the medium supports the growth of the desired organism; and, if a selective medium, it also inhibits the

Quality assessment		Quality control		
Internal quality assessment	External quality assessment	Evaluation and validation of tests	Internal quality control	Equipment evaluation and monitoring
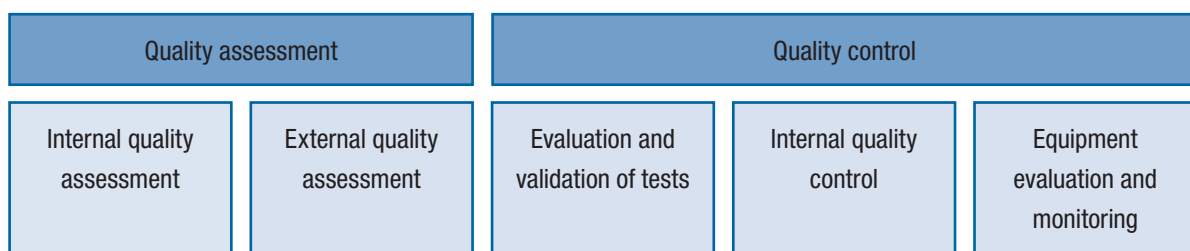
Figure 2.1
Different elements contributing to quality assurance (QA)

organisms not required. This will detect the absence or inadequate concentration of a growth factor or selective agent.

For molecular testing, IQC should include control sampling, control of nucleic acid isolation, an amplification control, a contamination control, and inhibition control. This will prevent false-negative polymerase chain reactions by detecting failure of one or more reagents, of amplification, of thermal cycling, or inhibition of amplification.

For any serological testing, QC samples should be included daily and include both the IQC supplied with the kit and external QC samples, where available, and tested in the same manner as the patient's specimens. The range for these QC samples for each laboratory should be established using at least 20 measurements throughout a period of time; the mean values, standard deviations (SD), and coefficient of variation (CV) then should be calculated. Levey–Jennings (LJ) plots should be prepared for each control and the QC results from each run compared. Comparisions should fall within two SDs, with results not reported if the QC value is greater than three SDs. These plots should be reviewed regularly to check for any abnormal findings or trends. Establishing a new range should be performed for each new batch of QC reagent.

Equipment evaluation and monitoring

Checking performance of the equipment on a regular basis and recording obtained data is essential in all laboratories (*6*). This can be achieved by maintaining an equipment inventory that is regularly reviewed. Microbiological safety cabinets should be monitored and recorded weekly for airflow; the temperature of all incubators, waterbaths, refrigerators, and freezers should be recorded daily. Any failures should be brought to the attention of the laboratory manager.

Quality assessment

Internal quality assessment (IQA)

IQA is performed by the periodical resubmitting and testing of randomly selected anonymised clinical samples within the same laboratory to ensure reproducibility of the results. All IQA specimens should undergo routine

analysis and receive no special treatment. For the scheme to provide both useful and relevant information, IQA is performed regularly for all sample types. Samples are selected by a trained member of staff, who must not be the same person who will undertake the testing. The samples should be selected randomly to prevent bias and should be reintroduced into the laboratory system in the same manner as normal samples.

The number of specimens tested and the frequency of testing is dependent on the total number of specimens received. A suggested number is approximately 1% should be retested on a monthly basis.

The results should be collated by an independent person and compared, with any discrepancies repeated and investigated.

External quality assessment (EQA)

EQA is performed by the distribution of proficiency panels by EQA providers such as United Kingdom National External Quality Assessment Service (UK NEQAS; http://www.ukneqas.org.uk), Quality Assessment Systems International (http://qasidirect.com), College of American Pathologists (http://www.cap.org), and the Australian RCPA Quality Assurance Programs Pty Ltd (http://www.rcpaqap.com.au), or as exchanges of samples between reference laboratories, to assess a broad range of techniques and assays performed in the clinical laboratory.

Suitably trained scientific staff should perform EQA and should treat it, as far as possible, according to normal laboratory practice. As EQA samples are usually clearly identifiable, there is the potential for them to be handled in ways that exceed normal laboratory procedures, e.g. handled by senior staff, repeat testing etc., and measures to avoid this should be put in place.

EQA provides a number of benefits for the laboratory:

- gives staff an insight into their laboratory's performance;
- compares the performance of the laboratory with that of other laboratories, nationally and/or internationally;
- improves the standard of examinations;
- identifies possible problem areas;

- demonstrates to clients, colleagues, and accreditation bodies that there is a commitment to quality;

- educates staff, providing a better understanding of the impact of incorrect results.

Processing of samples:

1. Samples should be reconstituted if required and where possible. This should be performed in a Class 1 Microbiology safety cabinet as risk is likely to be unknown.

2. Perform requested test or procedure according to the SOP.

3. Residual sample should be stored until results are known to enable tests to be repeated if a discrepancy has occurred.

4. When results are received from the external provider they should be recorded and the laboratory performance documented.

5. Any failures or discrepancies should be investigated by repeating the test.

6. Results should be reviewed by a senior scientist with experience and shared with laboratory staff, including successes and failures, to allow full discussion.

2.3 Quality indicators

Laboratories should consider setting indicators that reflect the quality of their results. Targets for turnaround times can be used to attempt to minimize the time from when the sample is taken to when the patient receives the results.

- A QMS is crucial to improve and maintain the accuracy and reproducibility of the results produced by a laboratory.
- All laboratories should strive to improve quality and work towards accreditation.
- An organizational structure and regular meetings of different levels of staff are necessary to review the quality system of the laboratory.
- Evaluation and validation of tests is essential to provide an evidence base before tests are used within a laboratory.
- QA encompasses QC and quality assessment.
- Laboratories should use indicators to monitor the quality of their laboratory tests.

2.4 References

1. Dobbs T et al. A comprehensive evaluation of the proficiency testing program for the HIV-1 BED incidence assay. *Journal of Clinical Microbiology*, 2011, 49(10):3470–3473.

2. Alemnji GA et al. Strengthening national laboratory health systems in the Caribbean Region. *Global Public Health*, 2012, 7(6):648–660.

3. Nkengasong JN et al. Laboratory systems and services are critical in global health: time to end the neglect? *American Journal of Clinical Pathology*, 2010, 134(3):368–373.

4. Yao K et al. Improving quality management systems of laboratories in developing countries: an innovative training approach to accelerate laboratory accreditation. *American Journal of Clinical Pathology*, 2010, 134(3):401–409.

5. Westerman LE et al. A quality management systems approach to CD4 testing in resource-poor settings. *American Journal of Clinical Pathology*, 2010, 134(4):556–567.

6. Fonjungo PN et al. Laboratory equipment maintenance: A critical bottleneck for strengthening health systems in sub-Saharan Africa. *Journal of Public Health Policy*, 2012, 33:34–45.

Chapter 3

Genital mycoplasmas

3.1 Introduction

Mycoplasmas is the trivial name for members of the class Mollicutes. Mycoplasmas are very small free-living bacteria usually ranging from 0.3 to 0.5 µm in size. They lack the rigid cell wall of other bacteria, making them resistant to penicillins and related antimicrobials. *M. genitalium* and *M. hominis* and the two ureaplasma species *U. urealyticum* (previously known as *U. urealyticum*, biovar 2) and *U. parvum* (previously known as *U. urealyticum*, biovar 1) are commonly found in the human urogenital tract. It is important to stress that before *U. urealyticum* and *U. parvum* were recognized as separate species, they were both designated *U. urealyticum*, making interpretation of the results of previous studies difficult. Table 3.1 presents disease associations.

M. genitalium is found in 1–3% of sexually active men and women in population-based studies. Ureaplasmas can be found in the cervix or vagina of 40–80% of sexually active, asymptomatic women, and *M. hominis* in 20–50% (*1–4*). Accordingly, ureaplasmas and *M. hominis* should be considered primarily as commensals when detected in the lower genital tract. However, these mycoplasmas are recognized as a cause of extragenital disease in patients with B-cell deficiencies (hypo- and agammaglobulinaemia) and in preterm infants (*5*).

M. genitalium has been strongly and uniformly associated with non-gonococcal urethritis (NGU) in more than 30 studies, and has been detected in the urethra of 15–25% of men with symptomatic NGU compared with about 5–10% of those without this disease (*1*). In studies that evaluated the association with non-chlamydial NGU (NCNGU), the association has generally been stronger, showing that *M. genitalium* and *C. trachomatis* act as separate causes of NGU. In several studies, *M. genitalium* has been found in more than one third of men with NCNGU (*1*). Among sexually transmitted disease (STD) clinic populations, approximately 90% of *M. genitalium*-infected men have microscopic signs of urethritis and almost three out of four report symptoms (*6, 7*).

Several clinical studies have shown a strong correlation between *M. genitalium* and persistent or recurrent NGU, probably due to the poor microbiologic treatment efficacy of tetracyclines. *M. genitalium* has generally been eradicated from less than one third of the infected patients after treatment with standard doses

Table 3.1: Disease associations of urogenital mycoplasmas

Species	Disease associations[a]						
	Urethritis	**Cervicitis**	**Bacterial vaginosis**	**Endometritis and/or PID**	**Preterm birth**	**Infertility (Women)**	**HIV transmission**
M. genitalium	++++	+++	–	+++	+/–	+	+
M. hominis	–	–	++++	+/–	+/–	–	ND
Ureaplasmas (undifferentiated)	+/–	–	+++	ND	+	+/–	ND
U. urealyticum	+	ND	ND	ND	ND	ND	ND
U. parvum	–	ND	ND	ND	ND	ND	ND

ND, not determined; PID, pelvic inflammatory disease.

[a] ++++ strong association, +++ association in most studies, + association only from a few studies, +/– conflicting results.

of tetracyclines (*8*). *M. genitalium* has been found in as many as 41% of men with persistent or recurrent urethritis after treatment with doxycycline (*9, 10*).

More recently, azithromycin treatment failure after a 1 g single dose was reported among 28% of men with *M. genitalium*-positive NGU and was correlated with macrolide resistance developed during treatment with a single dose in most patients (*11*).

In contrast with the consistency of studies associating *M. genitalium* with NGU, the role of the ureaplasmas in this disease has been more controversial and there is no evidence supporting a role for *M. hominis* as a cause of urethritis (*5*). Clearly, demonstrating ureaplasmas in a man with NGU does not necessarily indicate that this organism is the cause of the disease considering the high colonization rate. This is the case even if quantitative culture is applied. Thus, the exact proportion of cases for which ureaplasmas are responsible is unknown. The division of the human ureaplasmas into two species, *U. urealyticum* and *U. parvum*, led to studies suggesting that *U. urealyticum* may be associated with NGU in younger men with fewer partners (*12*) or when present in high titers. Thus, standard cultures that fail to discriminate between the species appear to be of very limited value.

M. genitalium has been associated with cervicitis, but the association is weaker than that between *M. genitalium* and male urethritis, possibly due to the difficulty and different criteria used in diagnosing cervicitis in women (*1*). However, in a number of studies, the association has been as strong as that for *C. trachomatis* (*7*). In studies where signs of urethritis have been reported, urethritis in women has been significantly associated with *M. genitalium* infection (*1*).

M. genitalium has been detected in the endometrium of 60% of women positive in the cervix, and its presence in endometrial biopsies has been strongly associated with histological endometritis and with recurrent pelvic inflammatory disease (PID) (*13, 14*). Tubal scarring has been indirectly linked with *M. genitalium* infection by a significantly higher proportion of women with tubal factor infertility having antibodies against the bacterium compared to women with infertility from other causes (*15*).

M. genitalium has been detected in the synovial fluid of a patient with sexually acquired reactive arthritis (SARA) (*16*) and clinical experience has shown that SARA is not uncommon after *M. genitalium*-positive NGU, but systematic studies have not been presented and the demonstration of *M. genitalium* in the synovial fluid has not been repeated. It has been shown that HIV-infected women with higher loads of *M. genitalium* organisms were more likely to shed HIV (*17*), but only recently has it been shown that *M. genitalium* infection predisposes to HIV acquisition (*18*). There is no indication that *M. hominis* or ureaplasmas may play a similar role.

- *M. genitalium* is a common cause of urethritis in men and women, and causes cervicitis and upper genital tract infection in women.
- *M. hominis* and ureaplasmas are commonly detected in healthy individuals. Their association with urogenital infection in either men or women remains to be conclusively proven.

3.2 Overview of available diagnostic methods for *M. genitalium*

For practical purposes, diagnosis of *M. genitalium* is limited to nucleic acid amplification test (NAAT), as culture is extremely slow (several months), challenging, and insensitive (*19*). To date, no serological assays, antigen detection assays, or point-of-care tests have proven useful for diagnosis of urogenital *M. genitalium* infections.

- NAAT is the only practical method for diagnosis of *M. genitalium*.

3.3 Collection, transport, and storage conditions of specimens

Sample collection should be performed as described for *C. trachomatis* (Chapter 5). Swabs and transport medium should not contain inhibitory substances for NAATs. It is also suitable to use a transport system that is compatible with the detection assay for *C. trachomatis*, as testing for this organism should have the highest priority. However, it should be noted that the organism load of *M. genitalium* is 100-fold lower than

that of *C. trachomatis* (*20*), so transport systems that dilute the specimen unnecessarily should be avoided. Unfortunately, no clear guidance can be given regarding the optimal specimen for detection of *M. genitalium*, as collection devices, sample preparation methods, and detection systems have varied between studies. If only one specimen is tested from each patient, it appears that first-void urine from men and vaginal swabs from women contain the highest load of bacteria.

3.4 Detection of *M. genitalium* by NAAT

Detection by NAAT is the only feasible method for detection of *M. genitalium*, but no commercially available assays have received United States of America Food and Drug Administration approval and most of the currently available assays have received very limited evaluation in published form. Most of those polymerase chain reaction (PCR) assays are based on detection of the MgPa adhesin gene of *M. genitalium* (*21*). Some parts of the MgPa gene, however, are highly variable and primers targeting these regions will not perform well with clinical specimens (*21, 22*). Although a single mismatch at several positions in the forward primer can be found in the forward primer with some strains, the MgPa TaqMan real-time assay described by Jensen et al. (*23*) has been widely used worldwide with good results. Also, the conventional MgPa PCR described as one of the first *M. genitalium* PCRs (*24*) or modifications of it (*25*) have proven very reproducible and have the advantage that the amplified fragment can be sequenced for a robust typing assay. The 16S rRNA gene also is used as target in *M. genitalium* PCRs, however, due to the homology between *M. genitalium* and *M. pneumoniae*, design of specific and sensitive primers and probes is relatively difficult.

For some 16S rRNA gene PCR assays, detection of *M. genitalium* is based on amplification with Mollicutes (mycoplasma and ureaplasma) universal primers and subsequent hybridization with species-specific probes. Although this approach allows detection of several mycoplasma species from the same primary amplification reaction, competition with amplification of ureaplasmal 16S rRNA gene sequences in particular will result in a poor sensitivity for detection of *M. genitalium* DNA, which may be significant even when found in low quantities (*21, 23*).

Several real-time *M. genitalium* PCR tests have been developed (*21*). Although well-optimized conventional PCRs may have the same sensitivity as well-optimized real-time assays, the real-time assays are less prone to contamination. Thus, the combination of high sensitivity, specificity, robustness, and reduced risk of contamination with amplicons, characteristics of this PCR format, suggests that real-time PCR should be the main method for *M. genitalium* diagnostics. Quantification of *M. genitalium* DNA does not appear to be of value for routine diagnostics.

As an alternative to PCR, a transcription-mediated amplification (TMA) assay targeting 16S rRNA has been available for research use only (Gen-Probe, San Diego, CA, USA). The advantage of this approach is the presence of multiple copies of 16S rRNA molecules per cell, leading to a potentially increased sensitivity of detection in comparison with the sensitivities of PCR assays that target single-copy genes. This TMA assay has been shown to be a sensitive, specific, and high-throughput test for *M. genitalium* detection, but too few studies have been performed to determine if it is superior to other NAATs (*26*).

In the absence of thoroughly validated commercially available NAATs, it is of utmost importance that laboratories performing *M. genitalium* diagnostics carefully validate and quality assure their in-house assays; see Chapters 1 and 2 and Annex 3 regarding NAATs and their quality assurance and validation of non-approved NAATs. In general, most multiplex assays suffer from some lack of sensitivity in diagnosing *M. genitalium* as this organism is often present at very low levels, even in symptomatic patients (*20, 23*).

- Sample preparation and assay sensitivity should be optimal for *M. genitalium* testing, as this pathogen is present in 100-fold lower concentrations than *C. trachomatis*. Most multiplex assays have a slightly lower sensitivity than assays with only one target.

- NAATs targeting the *M. genitalium* MgPa gene should be carefully designed to avoid variable regions of the gene.

Consequently, all efforts should be made to optimize the sample preparation step by using high-speed centrifugation for concentrating the specimen and to avoid loss of DNA during extraction.

3.5 Antimicrobial resistance and susceptibility testing of *M. genitalium*

Antimicrobial susceptibility testing of *M. genitalium* is very complicated and is only feasible in specialized reference laboratories. Broth dilution minimal inhibitory concentration (MIC) determination with a standardized inoculum is used as the reference method, but MIC determination of *M. genitalium* growing only in cell culture has also been successfully applied (*27*). Treatment failures of *M. genitalium* infections have led to an increased attention to resistance in this species. However, as *M. genitalium* is cultivable only in a few laboratories in the world, and since growth is too slow to allow meaningful results for the individual patient, molecular testing for the resistance-mediating mutations has been used in an increasing number of studies, and in daily clinical work in some countries.

Several clinical studies have shown that tetracylines are inferior to azithromycin in eradicating *M. genitalium* (*8*) and although in vitro MICs would suggest that this species should be tetracycline susceptible, clinical experience contradicts this. No molecular tests have been applied to detect tetracycline resistance. Macrolide resistance has been documented by isolation of *M. genitalium* strains from patients failing treatment with azithromycin (*28*) and the main resistance-mediating mutations have been shown to be in region V of the 23S rRNA gene, primarily A2058G and A2059G (*E. coli* numbering), but a range of other combinations has been detected in specimens from patients failing azithromycin therapy.

These mutations can be detected directly from clinical specimens by sequencing of PCR amplicons enabling clinically useful information in the absence of culture. It has been shown that treatment with azithromycin 1 g single dose leads to development of resistance or selection of pre-existing resistant variants (*28, 29*), and preliminary data suggest that the community level of macrolide resistance in *M. genitalium* is highly dependent on the use of azithromycin 1 g single dose for treatment

of *C. trachomatis* infection. This is illustrated by a low prevalence of resistance in countries using doxycycline as the primary drug for NGU and resistance as high as 100% in Greenland where azithromycin is used for NGU treatment and where the prevalence of *C. trachomatis* infections is extremely high (*30*). Moxifloxacin has proven to be an effective second-line treatment (*31*), but reports from Japan suggest that resistance can develop to the related fluoroquinolone gatifloxacin (*32*), and strains with combined high-level quinolone and macrolide resistance have been isolated (J.S. Jensen, unpublished data, April 2013). Although mutations in the quinolone resistance-determining regions have been found (*32*), their clinical correlates have not been determined.

- Macrolide resistance in *M. genitalium* is very common in patients failing treatment with azithromycin, and moxifloxacin is the most frequently used second-line treatment.

- Macrolide resistance in *M. genitalium*, mediated by specific mutations in the 23S rRNA gene, can be detected by PCR-amplifying and sequencing this gene directly from clinical specimens.

- Mutations in the *M. genitalium* quinolone resistance-determining regions have been found in patients failing quinolone treatment, but their clinical correlates have not been established.

3.6 References

1. Taylor-Robinson D, Jensen JS. *Mycoplasma genitalium*: from chrysalis to multicolored butterfly. *Clinical Microbiology Reviews*, 2011, 24(3):498–514.

2. Andersen B et al. *Mycoplasma genitalium*: prevalence and behavioural risk factors in the general population. *Sexually Transmitted Infections*, 2007, 83(3):237–241.

3. Manhart LE et al. *Mycoplasma genitalium* among young adults in the United States: an emerging sexually transmitted infection. *American Journal of Public Health*, 2007, 97(6):1118–1125.

4. McCormack WM et al. Sexual activity and vaginal colonization with genital mycoplasmas. *Journal of the American Medical Association*, 1972, 221(12):1375–1377.

5. Totten PA, Taylor-Robinson D, Jensen JS. Genital mycoplasmas. In: Holmes KK et al., eds. *Sexually

transmitted diseases, 4th ed. New York, McGraw-Hill Medical, 2008:709–736.

6. Falk L, Fredlund H, Jensen JS. Symptomatic urethritis is more prevalent in men infected with *Mycoplasma genitalium* than with *Chlamydia trachomatis. Sexually Transmitted Infections*, 2004, 80(4):289–293.

7. Anagrius C, Loré B, Jensen JS. *Mycoplasma genitalium*: prevalence, clinical significance, and transmission. *Sexually Transmitted Infections*, 2005, 81(6):458–462.

8. Manhart LE, Broad JM, Golden MR. *Mycoplasma genitalium*: should we treat and how? *Clinical Infectious Diseases*, 2011, 53(Suppl 3):S129–S142.

9. Horner P et al. Role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* in acute and chronic nongonococcal urethritis. *Clinical Infectious Diseases*, 2001, 32(7):995–1003.

10. Wikström A, Jensen JS. *Mycoplasma genitalium*: a common cause of persistent urethritis among men treated with doxycycline. *Sexually Transmitted Infections*, 2006, 82(4):276–279.

11. Bradshaw CS et al. Azithromycin failure in *Mycoplasma genitalium* urethritis. *Emerging Infectious Diseases*, 2006, 12(7):1149–1152.

12. Wetmore CM et al. *Ureaplasma urealyticum* is associated with nongonococcal urethritis among men with fewer lifetime sexual partners: a case-control study. *Journal of Infectious Disease*s, 2011, 204(8):1274–1282.

13. Cohen CR et al. Association between *Mycoplasma genitalium* and acute endometritis. *The Lancet*, 2002, 359(9308):765–766.

14. Haggerty CL et al. Failure of cefoxitin and doxycycline to eradicate endometrial *Mycoplasma genitalium* and the consequence for clinical cure of pelvic inflammatory disease. *Sexually Transmitted Infections*, 2008, 84(5):338–342.

15. Svenstrup HF et al. *Mycoplasma genitalium*, *Chlamydia trachomatis*, and tubal factor infertility—a prospective study. *Fertility and Sterility*, 2008, 90(3):513–520.

16. Taylor-Robinson D et al. *Mycoplasma genitalium* in the joints of two patients with arthritis. *European Journal of Clinical Microbiology and Infectious Diseases*, 1994, 13(12):1066–1069.

17. Manhart LE et al. High *Mycoplasma genitalium* organism burden is associated with shedding of HIV-1 DNA from the cervix. *Journal of Infectious Diseases*, 2008, 197:733–736.

18. Mavedzenge SN et al. The association between *Mycoplasma genitalium* and HIV-1 acquisition in African women. *AIDS*, 2012, 26(5):617–624.

19. Jensen JS, Hansen HT, Lind K. Isolation of *Mycoplasma genitalium* strains from the male urethra. *Journal of Clinical Microbiology*, 1996, 34(2):286–291.

20. Walker J et al. The difference in determinants of *Chlamydia trachomatis* and *Mycoplasma genitalium* in a sample of young Australian women. *BMC Infectious Diseases,* 2011, 11:35.

21. Shipitsyna E et al. Guidelines for the laboratory diagnosis of *Mycoplasma genitalium* infections in East European countries. *Acta Dermatovenereologica*, 2010, 90(5):461–467.

22. Ma L et al. Genetic variation in the complete MgPa operon and its repetitive chromosomal elements in clinical strains of *Mycoplasma genitalium. PLoS One*, 2010, 5(12):e15660.

23. Jensen JS et al. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *Journal of Clinical Microbiology*, 2004, 42(2):683–692.

24. Jensen JS et al. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *Journal of Clinical Microbiology*, 1991, 29(1):46–50.

25. Dutro SM et al. Development and performance of a microwell-plate-based polymerase chain reaction assay for *Mycoplasma genitalium. Sexually Transmitted Diseases*, 2003, 30(10):756–763.

26. Wroblewski JK et al. Comparison of transcription-mediated amplification and PCR assay results for various genital specimen types for detection of *Mycoplasma genitalium. Journal of Clinical Microbiology*, 2006, 44(9):3306–3312.

27. Hamasuna R, Osada Y, Jensen JS. Antibiotic susceptibility testing of *Mycoplasma genitalium* by TaqMan 5' nuclease real-time PCR. *Antimicrobial Agents and Chemotherapy*, 2005, 49(12):4993–4998.

28. Jensen JS et al. Azithromycin treatment failure in *Mycoplasma genitalium*-positive patients with nongonococcal urethritis is associated with induced macrolide resistance. *Clinical Infectious Diseases*, 2008, 47(12):1546–1553.

29. Twin J et al. Transmission and selection of macrolide resistant *Mycoplasma genitalium* infections detected by rapid high resolution melt analysis. *PLoS One*, 2012, 7(4):e35593.

30. Gesink DC et al. *Mycoplasma genitalium* presence, resistance and epidemiology in Greenland. *International Journal of Circumpolar Health*, 2012, 71:1–8.

31. Bradshaw CS, Chen MY, Fairley CK. Persistence of *Mycoplasma genitalium* following azithromycin therapy. *PLoS One*, 2008; 3(11):e3618.

32. Hamasuna R et al. P3–S7.09 mutations on *gyrA* or *parC* genes of *Mycoplasma genitalium* and efficacies of treatment with fluoroquinolones against *M genitalium*-related urethritis. *Sexually Transmitted Infections*, 2011, 87(Suppl. 1):A302.

Chapter 4

Gonorrhoea

4.1 Introduction

Gonorrhoea, caused by *Neisseria gonorrhoeae* (gonococcus), is an ancient disease that is transmitted almost exclusively through sexual contact. In 2008, according to World Health Organization (WHO) estimates, there were 106 million new cases among adults globally. This places gonorrhoea as the most prevalent bacterial sexually transmitted infection (STI) together with *Chlamydia trachomatis* infection (also 106 million new cases) (*1*). Accordingly, gonorrhoea, including its severe complications, causes substantial morbidity and economic costs, and remains a major public health concern globally. It is of grave concern that the bacterium has now developed resistance to nearly all antimicrobials introduced for treatment of gonorrhoea, and it is feared that gonorrhoea may become untreatable in certain circumstances (*2*, *3*).

The genus Neisseria contains two species primarily pathogenic to humans, *N. gonorrhoeae* and *N. meningitidis*, and approximately 30 usually nonpathogenic species such as *N. lactamica*, *N. sicca*, *N. cinerea*, *N. flavescens*, *N. subflava*, and *N. mucosa*. These organisms predominantly inhabit the upper respiratory tract as commensals, but may be found infrequently in the lower urogenital tract. Gonococci are Gram-negative, aerobic, capnophilic (prefer enhanced concentration [3–7%] of carbon dioxide [CO_2]), non-flagellated, non-sporulating, and oxidase- and catalase-producing cocci, which typically are arranged in pairs (diplococci) with adjacent sides concave, i.e. in microscopy they appear in a characteristic kidney or coffee bean morphology. *N. gonorrhoeae* is fastidious and requires complex nutritionally enriched culture medium for in vitro growth.

N. gonorrhoeae infects humans only, colonizes mucosal surfaces, and is the etiological agent of lower urogenital tract infections—urethritis in men and cervicitis in women. Asymptomatic urogenital infection occurs in a minority of men but is more common (at least 50%) in women. Infection of the rectum (proctitis) and the pharynx, commonly asymptomatic, can occur in both sexes depending on sexual behaviour but is predominantly found in men who have sex with men (MSM). If undetected and not treated or inappropriately treated, infection can ascend to the upper genital tract and cause complicated gonococcal infection (e.g. pelvic inflammatory disease [PID] and related sequelae such as ectopic pregnancy and infertility) in women, and penile oedema and epididymitis in men. Conjunctivitis can occur in adults but, most commonly, infection of the eye presents as ophthalmia neonatorum in the newborn. Disseminated gonococcal infection (DGI), which is a distinct entity and not a true complication, can occur in both sexes but is rarely encountered. Table 4.1 summarizes the clinical manifestations of gonococcal infections.

- *N. gonorrhoeae* causes the most common bacterial STI globally (in 2008, exactly as common as chlamydial infection), which includes a spectrum of diseases in a variety of sites, including urogenital, pharyngeal, rectal, and conjunctival.

- Complications and sequelae associated with untreated *N. gonorrhoeae* infections include PID, ectopic pregnancy, infertility, penile oedema, epididymitis, and DGI.

4.2 Overview of available diagnostic methods

Gonorrhoea is frequently asymptomatic, especially in women, and in the pharynx, and rectum, and symptoms, if present, can be non-specific (see Table 4.1). Accordingly, laboratory procedures are needed for diagnosis, case-finding, and test-of-cure. The diagnosis of gonorrhoea is established by identification of *N. gonorrhoeae* in genital or extra-genital secretions.

Table 4.1: Clinical manifestations of gonococcal infections[a]

Uncomplicated gonorrhoea[a]	
Urethra	• Copious, purulent discharge • Scant, clear discharge • Dysuria
Cervix	• Red, friable cervical os • Purulent discharge from os • Dysuria • Salpingitis • Bilateral or unilateral lower abdominal tenderness
Rectum	• Copious, purulent discharge • Burning/stinging pain • Tenesmus • Blood in stools
Pharynx	• Mild pharyngitis • Mild sore throat • Erythema
Conjunctiva	• Copious, purulent discharge • Keratitis and corneal ulceration; perforation, extrusion of lens • Scarring; opacification of lens • Blindness

Complicated gonorrhoea[b]	
Male complications	• Penile oedema • Tyson's glands abscess • Cowper's glands abscess • Seminal vesiculitis • Epididymitis • Infertility (rare)
Female complications	• Endometritis • Salpingitis • Bartholin abscess • Lymphangitis • Tubo-ovarian abscess • Ectopic pregnancy • Infertility
Disseminated gonococcal infection (DGI)	• Bacteremia • Fever • Dermatitis (skin lesions: macular, erythematous, pustular, necrotic, haemorrhagic) • Tenosynovitis • Joints; septic arthritis • Endocarditis • Meningitis

[a] As noted above, gonococcal infection may be asymptomatic, particularly in women, and in the pharynx and rectum.
[b] Epidemiological and biological studies provide strong evidence that gonorrhoea significantly facilitates HIV transmission.

Table 4.2 summarizes the recommended methods for the diagnosis of gonorrhoea and the performance and other characteristics of these methods.

A correctly prepared, Gram-stained, and microscopy-examined smear to identify Gram-negative intracellular diplococci in polymorphonuclear leukocytes (PMNL) is sensitive (95%) and specific (97%) for the diagnosis of gonorrhoea in symptomatic men with urethral discharge. In women, however, smears of cervical secretions detect only 40–60% of culture-positive specimens, which may reflect the lower number of gonococci in women. False-positive results can occur, and the specificity (80–95%) is dependent on the experience of the microscopist. Direct microscopic examination is not recommended for the diagnosis of rectal and pharyngeal infections because of the large number of other organisms present and the low sensitivity. Screening of asymptomatic individuals by microscopy is not recommended.

For many decades, culture of *N. gonorrhoeae* has been considered the "gold standard" for the diagnosis of both genital and extra-genital gonorrhoea. Culture is sensitive and highly specific in optimized circumstances, is inexpensive, and importantly allows antimicrobial susceptibility testing. Antimicrobial resistance in gonococci is a severe problem worldwide and, as culture is the only method allowing antimicrobial susceptibility testing, it is crucial to maintain and, where necessary, strengthen the culture capacity in all countries.

Throughout the last two decades, nucleic acid amplification tests (NAATs) have been developed and introduced for detection of specific *N. gonorrhoeae* DNA/RNA. These are generally more sensitive than culture for

Table 4.2: Common diagnostic tests (as of June 2012) for detection of *N. gonorrhoeae*

	Microscopy[a]	Culture	NAAT
Specimen types			
Endocervical swab	Yes[a]	Yes	Yes
Vaginal swab	No	Yes[b]	Yes (some assays)

Urine
  Female
  Male
	
	No
	No
		
		No
		No
			
			Yes[c]
			Yes

Urethral swab	Yes[a]	Yes	Yes
Rectal swab	No	Yes	No[d]
Oropharyngeal swab	No	Yes	No[d]
Conjunctival swab	Yes	Yes	No[d]
Performance			
Sensitivity[e]	Low–high[a]	Moderate–high	Very high
Specificity[e]	Moderate–high[a]	Very high	Moderate–very high
Other considerations			
Cost	Low	Moderate	High–very high
Instrumentation	Microscope	Routine microbiology	Large footprint
Throughput/automation	Moderate/no	Moderate/no	High/possible
Technical complexity	Low	Moderate	High
Level of laboratory infrastructure	Peripheral	Peripheral–intermediate	Intermediate–central
Multiple pathogens from one sample	No	No	*C. trachomatis*, *T. vaginalis*, and HPV on some platforms
Other comments		• Strict sample collection, transportation, and storage are crucial to maintaining viability • This is the only method that allows antimicrobial susceptibility testing.	• NAATs generally have a superior sensitivity compared to culture, especially for pharyngeal and rectal samples. However, the specificity can be suboptimal, and confirmation using supplementary NAAT may be required.

HPV, human papillomavirus; NAAT, nucleic acid amplification test.

[a] Microscopy has high sensitivity and specificity in symptomatic men (with urethritis), low sensitivity in asymptomatic men, and endocervical infections, and is not recommended for vaginal, urine, rectal, or pharyngeal specimens.

[b] Not an ideal specimen, mainly applied for prepubertal girls or women who have had a hysterectomy.

[c] Urine is not the ideal sample, due to suboptimal sensitivity, for detection of *N. gonorrhoeae* in women.

[d] There are no internationally licensed NAAT for use with extra-genital samples, but there is increasing evidence that NAATs are more sensitive than culture at these sites. It is recommended that a positive NAAT test for rectal and pharyngeal specimens be confirmed with a supplementary test (NAAT with another target sequence) to avoid false-positive results.

[e] Sensitivity and specificity estimates vary widely depending on the different sensitivity and specificity of assays of the same methodology as well as assays used for comparison (the "gold standard").

the diagnosis of gonorrhoea, especially for pharyngeal and rectal samples (*4–6*).

However, a commercially available NAAT with United States of America Food and Drug Administration (FDA) approval is not yet licensed for use on pharyngeal or rectal specimens. Furthermore, the specificity of several of the gonococcal NAATs has proven suboptimal in the diagnostics of urogenital samples and, in particular, extra-genital samples, which results in low positive predictive value (PPV), in particular, in low prevalence populations.

To date there are no direct immunofluorescence assays, enzyme immunoassays, or rapid point-of-care tests for antigen detection with appropriate performance characteristics (sensitivity and specificity) available for the diagnosis of uncomplicated or complicated gonorrhoea. Furthermore, there is no commercial and internationally approved method for detection of gonococcal antibodies in serum, and these methods cannot differentiate current from past infection. These methods should not be used due to their suboptimal sensitivity and specificity for the diagnosis of uncomplicated or complicated gonorrhoea in individual patients.

For appropriate performance characteristics of all diagnostic methods, it is crucial to follow precisely the standard operating procedures for performance and recommendations from the manufacturer with regard

- Microscopy is sensitive and specific in symptomatic men with urethral discharge.

- Culture is sensitive and highly specific in optimized circumstances, inexpensive, and allows antimicrobial susceptibility testing.

- NAATs generally have a superior sensitivity compared to culture, especially for pharyngeal and rectal samples. However, the specificity of several of the gonococcal NAATs is suboptimal in the diagnostics of urogenital samples and, in particular, extra-genital samples, which results in low PPVs in low prevalence populations.

to collection, transportation, and storage of samples, as well as performance, including quality control (QC) of the specific assay.

4.3 Collection, transportation, and storage of specimens

Appropriate anatomical sites for specimen collection depend on the sex, age, and sexual behaviour of the individual; clinical manifestations of the infection; and diagnostic testing method, including its performance characteristics (sensitivity and specificity). The primary collection site in women using culture and microscopy is the endocervical canal; for NAATs, is the endocervical canal or vagina. The secondary sites include the urethra, rectum, and oropharynx. In heterosexual men, specimens for culture and microscopy should be collected from the urethra, and for NAATs, a urine specimen. In MSM and men and women with indicative clinical signs and/ or sexual practice (oral and/or anal sex), the rectum and oropharynx should be sampled additionally. NAATs have higher sensitivity for these infections than culture and can increase case finding (*4–6*), although, as noted, no commercial kit is licensed for these sites. For culture, which requires live organisms, the sample must be collected from sites with columnar or cuboidal epithelial cells, and charcoal-coated (if charcoal is not included in the non-nutritive tranport medium) sterile Dacron or rayon swabs are ideally used. For diagnostics using NAATs, recommendations from the manufacturer in regard to collection, transportation, and storage of samples must be followed in detail. The use of antiseptics, analgesics, and lubricants when collecting specimens should be avoided since these may inhibit gonococci. All specimen collection should be performed before initiating antimicrobial treatment.

Endocervix: Insert a swab 2–3 cm into the cervical os and rotate gently for 5–10 seconds. Endocervical samples should not be taken in prepubertal girls or women who have had a hysterectomy; instead, specimens should be sampled from the vestibule of the vagina and a urine specimen (for NAAT diagnostics) should also be sampled.

Urethra: Take urethral specimens at least 1 hour after the patient has urinated. Collect discharge directly on a

swab. If no discharge is evident, in men the urethra is stripped towards the orifice to evacuate exudate. If no exudate is obtained, insert a thin swab 2–3 cm into the urethra and gently rotate for 5–10 seconds. In women, massage the urethra against the pubic symphysis and use the same technique as for men. Culturing both endocervix and urethra when testing women can increase case finding.

Vagina (NAATs only): The swab should be rotated against the posterior vaginal walls for 5 seconds. Vaginal swabs may be patient- or clinician-obtained with equal utility (*7, 8*).

First void (catch) urine (NAATs only): Do not have the patient clean the genital area. Catch 10–20 ml of first void urine in a sterile collection container at least 1 hour after the patient has urinated.

Rectum: Insert a swab 2–3 cm into the rectum and rotate it against all the rectal walls for 10 seconds. If faecal contamination occurs, discard the swab and use another to obtain the specimen. In symptomatic patients, anorectal specimens should be obtained ideally under direct vision following insertion of a proctoscope.

Oropharynx: Swab the region of the posterior pharynx above the inferior edge of the soft palate and the tonsillar crypts.

Conjunctiva: Retract the inferior eyelid and move a thin swab across the surface of the inferior palpebral conjunctiva towards the median corner of the eye.

4.4 Presumptive diagnosis: microscopy

4.4.1   Preparation of slides for staining

Prepare the smear as described in Annex 1 (Microscopy). Fix the dried smear by heating either on a hotplate or by passing the slide rapidly through a flame three times,

while keeping the film side uppermost. Avoid overheating since this distorts the cells. The slide should feel merely warm when touched with the back of the wrist.

Apart from being simple and quick, microscopy after staining with methylene blue (Fig. 4.1) is a reliable method for the diagnosis of gonorrhoea in men with purulent urethritis (*9*) but does not allow differentiation of Gram-negative cocci and therefore lacks ideal specificity. The Gram stain to identify intracellular Gram-negative diplococci within PMNL is the method of choice for the presumptive diagnosis of *N. gonorrhoeae* (Fig. 4.2). Extracellular organisms may be observed, but they alone are insufficient for diagnosis but are sometimes used in combination with clinical symptoms.
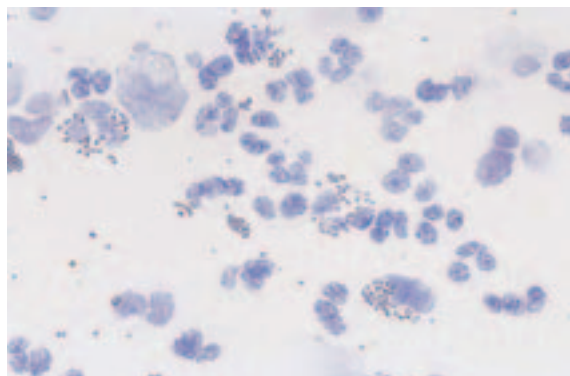
Figure 4.1
Microscopy of methylene blue stain of a male urethral exudate showing intracellular diplococci within PMNL (1000×)
Source: Reproduced from Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010.
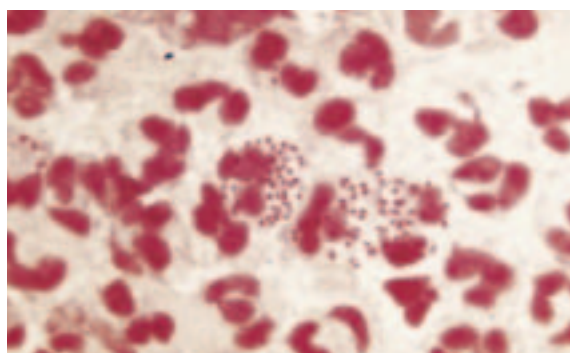
Figure 4.2
Microscopy of Gram stain of a male urethral exudate showing Gram-negative intracellular diplococci within PMNL (1000×)
Source: Reproduced from Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010.

Table 4.3: Sample collection, transportation, and storage

Anatomic site	Collection device	Sampling procedure	Microscopy	Culture	NAAT
Endocervix	Swab/plastic[a] (OR endocervical brush OR assay-specific collection kit for NAATs)	Use a vaginal speculum and clean the ectocervix. Insert swab 2–3 cm and rotate for 5–10 seconds.	Roll onto slide (thin layer) and air dry (see Annex 1). The sensitivity for endocervical samples is suboptimal.	Bed-side inoculation should be performed on selective gonococcal medium and incubated immediately. If bed-side collection is not performed, gonococcal non-nutritive transport medium or nutritive transport medium should be used.[b] Specimens in non-nutritive transport medium should be inoculated at laboratory as soon as possible and within 48 hours at the latest (see also section 4.5).	Place into manufacturer's collection device. Transport and store according to manufacturer's instructions. If transport medium is not available from manufacturer, use appropriate transport medium stabilizing the nucleic acid, e.g. GeneLock tubes.
Urethra (collected ≥1 hour after last void)	Swab/aluminium[c] (OR assay-specific collection kit for NAATs)	Collect discharge directly on a swab. Insert swab 2–3 cm into the urethra and gently rotate for 5–10 seconds.	Roll onto slide (thin layer) and air dry (see Annex 1).	Transportation and storage, see endocervical sample for culture.	Transportation and storage, see endocervical sample for NAAT.
Vagina	Swab/plastic[a] (OR assay-specific collection kit for NAATs)	Clinicians or patients can obtain samples. Rotate swab against all posterior vaginal walls for 5 seconds.	NA	Used for prepubertal girls or women who have had a hysterectomy. Transportation and storage, see endocervical sample for culture.	Transportation and storage, see endocervical sample for NAAT.[d]
Urine (collected ≥1 hour after last void)	Sterile urine cup	Patient should not clean the genital area. Catch first void urine (less than 25 ml in general).	NA	NA	Transportation and storage, see endocervical sample for NAAT.

Table 4.3: Sample collection, transportation, and storage (*continued*)

Anatomic site	Collection device	Sampling procedure	Microscopy	Culture	NAAT
Rectum	Swab/plastic[a] (OR assay-specific collection kit for NAATs)	Insert swab 2–3 cm into the rectum and rotate it against all the rectal walls for 10 seconds.	NA	Transportation and storage, see endocervical sample for culture.	Transportation and storage, see endocervical sample for NAAT.[d]
Oropharynx	Swab/plastic[a] (OR assay-specific collection kit for NAATs)	Swab the posterior pharynx and the tonsillar crypts.	NA	Transportation and storage, see endocervical sample for culture.	Transportation and storage, see endocervical sample for NAAT.[d]
Conjunctiva	Swab/ aluminium[c] (OR assay-specific collection kit for NAATs)	Purulent discharge should be removed with a swab. Retract the inferior eyelid. Swab the surface of the inferior palpebral conjunctiva.	Roll onto slide (thin layer) and air dry (see Annex 1). Mainly for neonates.	Transportation and storage, see endocervical sample for culture.	Transportation and storage, see endocervical sample for NAAT.[d]

NA, not applicable; NAAT, nucleic acid amplification test.

[a] Dacron or rayon swabs on a plastic shaft.

[b] Appropriate *N. gonorrhoeae* non-nutritive transport medium such as Amies or Stuart (stored at 4°C before transport), or nutritive (growth) transport medium like Jembec, Transgrow, Gono-Pak, or InTray GC system (stored at 36±1°C before transport) should be used.

[c] Dacron or rayon swabs on an aluminium shaft.

[d] No gonococcal NAATs are approved yet by the United States of America Food and Drug Administration (FDA) for extra-genital samples; however, for rectal and oropharyngeal specimens, appropriate NAATs have proven more sensitive than culture (*4–6*), and these may be used if appropriate validation data exists.

4.4.2 Gram stain procedure

1. Cover the fixed smear with crystal violet for 30 seconds. Gently rinse with cold tap water.

2. Flood the slide with iodine solution for 30 seconds. Gently rinse with cold tap water.

3. Decolorize with acetone, acetone-ethanol, or ethanol alone until the purple colour stops flooding out of the smear. It is best to hold the slide in a gloved hand near running water. The time of decoloration will depend on which chemical agent is used and the thickness of the smear—it will be shortest (typically a few seconds) for acetone and require longer (up to a minute) for ethanol. Excessive decoloration must be avoided; otherwise Gram-positive bacteria appear as Gram-negative. Disregard the thick portions of an uneven smear, which may stain blue.

4. Rinse quickly under running water to stop the decoloration and drain off excess water.

5. Counterstain with safranin or fuchsin for 1 minute.

6. Rinse with running water and gently blot the slide with absorbent paper.

4.4.3 Methylene blue stain procedure

1. Cover the smear with methylene blue stain for 30 to 60 seconds.

2. Rinse with running water and gently blot the slide with absorbent paper.

4.4.4 Smear reading and interpretation

Use a bright-light microscope and immersion oil of good quality, and examine the slide with a 100× objective (10× ocular). Gonococci appear as Gram-negative diplococci within PMNL. Always describe exactly what is seen on the smear: epithelial cells, PMNL, morphologies of bacteria, and intracellular or extracellular location. A slide should be examined for at least 2 minutes before concluding it does not contain any Gram-negative intracellular diplococci.

4.4.5 Quality control (QC) of microscopy using Gram-stained smears

QC should be performed at regular intervals using a range of bacteria giving different Gram reactions and/ or control specimens. This should always be performed when using a new batch of reagents.

- Microscopy is sensitive and specific in symptomatic men with urethral discharge.

- However, microscopy has a lower sensitivity in asymptomatic men and endocervical infections, and does not provide a definitive diagnosis for these infections.

- Microscopy is not recommended for the diagnosis of rectal and pharyngeal infections.

4.5 Culture and identification of *N. gonorrhoeae* (presumptive and definitive)

4.5.1 Transport and culture

Culture remains essential for antimicrobial susceptibility testing (see section 4.8). Gonococci are highly susceptible to environmental conditions (temperature, desiccation, oxidation, and toxic substances) and transportation of specimens from the clinic to the laboratory (Table 4.3) will reduce the viability of the organisms. Specimens inoculated directly onto a nutritious selective culture medium (see below) in the consultation room is the optimal method, but if this is not feasible, the swabs should be inserted into a non-nutritive transport medium such as Stuart or Amies (see Annex 4) or inoculated on a nutritive (growth) transport system such as Transgrow (*10*, *11*), Jembec (*11*, *12*), Gono-Pak, or InTray GC system. Using a non-nutritive transport medium, the isolation rate after transportation of specimens at room temperature (20–25°C) is approximately 100% within 6 hours and more than 90% within 12 hours.

After 48 hours, however, the number of gonococci decreases and recovery may no longer be possible, especially in specimens from asymptomatic patients that contain small numbers of organisms. When a transit time of more than 48 hours is expected, nutritive (growth) transport systems that incorporate a culture medium and provide an atmosphere with enhanced concentration of CO_2 ideally are used. Maximum survival and recovery of gonococci from non-nutritive transport medium is obtained when the inoculated Stuarts or Amies transport medium (see Annex 4) are stored in the refrigerator at 2–8°C before transportation to the laboratory. These media retard the growth of gonococci, preventing loss of viability. In contrast, maximum survival from nutritive (growth) transport media is obtained when the specimens are pre-incubated in the transport medium at 36±1°C overnight before transportation to the laboratory; acceptable results are obtained if transport time does not exceed 2 days (*11*).

The reliability of a culture for the diagnosis of gonorrhoea is conditional on a series of factors:

- number of sampling sites

- technique and swabs used for collection of specimens

- conditions and duration of transportation

- composition and quality of the culture medium

- inoculation and incubation conditions

- reagents and techniques used for the species identification of *N. gonorrhoeae*.

The prevalence of gonococcal strains susceptible to the concentration of antimicrobials generally used in selective culture agar media is negligible in most countries. As a result, it is recommended that nutritious selective culture media such as the Thayer–Martin (*13*), modified Thayer–Martin media (MTM; see Annex 4) and New York City agar (NYC) (*14*) are used for routine diagnosis of gonorrhoea. If resources allow, for urogenital specimens, it is ideal to use also a non-selective culture plate for each sample. In some geographical areas, up to 5% of gonococci can be susceptible to the 3–4 mg/l concentration of vancomycin used in the selective culture media (*15*); therefore, selective supplements with a reduced concentration of vancomycin of 2 mg/l or with lincomycin (1 mg/l), which is less inhibitory to contaminating bacteria than vancomycin, are recommended. If rectal and pharyngeal samples are routinely tested, the high-level concentration of vancomycin is advisable.

4.5.2 Quality control (QC) of culture media

Each batch of medium should be controlled for sterility, its ability to sustain the growth of gonococci, and its ability to inhibit other contaminating microorganisms. For evaluation of growth ability and QC of all diagnostic methods, the 2008 WHO *N. gonorrhoeae* reference strains (*16*) are available from WHO sources (see section 4.8.3.3). For controlling the inhibition of non-gonococcal microorganisms' reference strains of, for example, *Escherichia coli* (ATCC 25922), *Staphylococcus epidermidis* (ATCC 12228), *N. sicca* (ATCC 9913), and *Candida albicans* (ATCC 14053) can be used.

4.5.3 Culture inoculation and incubation

Roll the swab containing the specimen over approximately one quarter of the surface of the plate, ideally Petri dishes with a diameter of 90 mm. Using a sterile bacteriological loop, spread the inoculum over the remaining part of the medium to ensure growth of isolated colonies. Alternatively, the specimen can be inoculated over the entire surface of the plate in a 'Z' pattern and then streaked out; this can yield more isolated colonies. Incubate the inoculated plates immediately at $36\pm1°C$ in a humid atmosphere (approximately 70–80% humidity) containing $5\pm1\%$ CO_2 (candle extinction jar with moistened cotton wool ball or towels [Fig. 4.3], jar with CO_2-generating envelopes, or CO_2 incubator with water bowl or other equipment for enhanced humidity). Examine the plates after 18–24 hours, and—if negative—again after 48 hours. After 24 hours of incubation, typical colonies may vary in diameter from 0.5 to 1 mm, and in appearance from grey to white, transparent to opaque, and convex to flat (depending on gonococcal strain and culture medium; Fig. 4.4). After further incubation, they may reach 3 mm in diameter and become less smooth. Frequently, a mixture of different colony types appears on a plate.

Figure 4.3
Candle extinction jar for incubation of
***N. gonorrhoeae* culture plates**
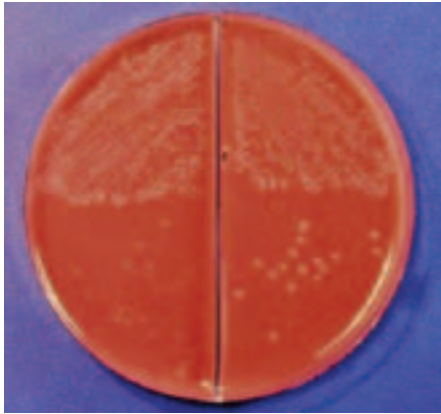
Figure 4.4
Typical colonies of *N. gonorrhoeae* **on gonococcal selective (left: MTM) and non-selective culture agar media (right: MTM without antimicrobials added) showing slight growth inhibition by the selective antimicrobials**
Source: Reproduced from Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010.

4.5.4 Presumptive identification of *N. gonorrhoeae* after culture

A presumptive identification of colonies with a gonococcus-like appearance on selective media can be made by a Gram stain (see section 4.4.2) and an oxidase test. An oxidase test detects the presence of cytochrome c oxidase; the test is best performed by rubbing a few colonies directly onto a filter paper strip moistened with the reagent (1% aqueous solution of tetramethyl-para-phenylenediamine dihydrochloride)

prepared either in-house or commercially (e.g. BACTIDROP oxidase). A positive test changes in colour from colourless to purple in a few (maximum of 30) seconds (Fig. 4.5). Alternatively, the oxidase test can be performed by placing a drop of oxidase reagent on a few representative colonies on a pure growth culture. Care should be taken as the reagent is toxic to the bacteria and, if only a few colonies are present, they should be subcultured before testing (Fig. 4.5). The observation of oxidase positive, Gram-negative diplococci with typical colonial morphology on selective media from genital specimens offers a sufficient and reliable identification of *N. gonorrhoeae* for presumptive diagnosis and is highly predictive of *N. gonorrhoeae* if grown on gonococcal-selective agar from a high-risk patient. In situations where resources are limited, this is sufficient to initiate treatment. However, to provide a definitive diagnosis of gonorrhoea, it is necessary to confirm the identification of *N. gonorrhoeae* by eliminating closely related species, such as *N. meningitidis*, *N. lactamica*, and *N. cinerea*, that also might grow on the gonococcal primary isolation selective media. It is advisable always to confirm the identification of Gram-negative, oxidase-positive colonies from all extragenital sites, as the likelihood of isolating Neisseria species other than *N. gonorrhoeae* at these sites is higher, particularly in the pharynx. Any gonococcal isolates that are to be further characterized, e.g. by antimicrobial susceptibility testing (see section 4.8) or by phenotypic or genetic typing should also have their identification confirmed.
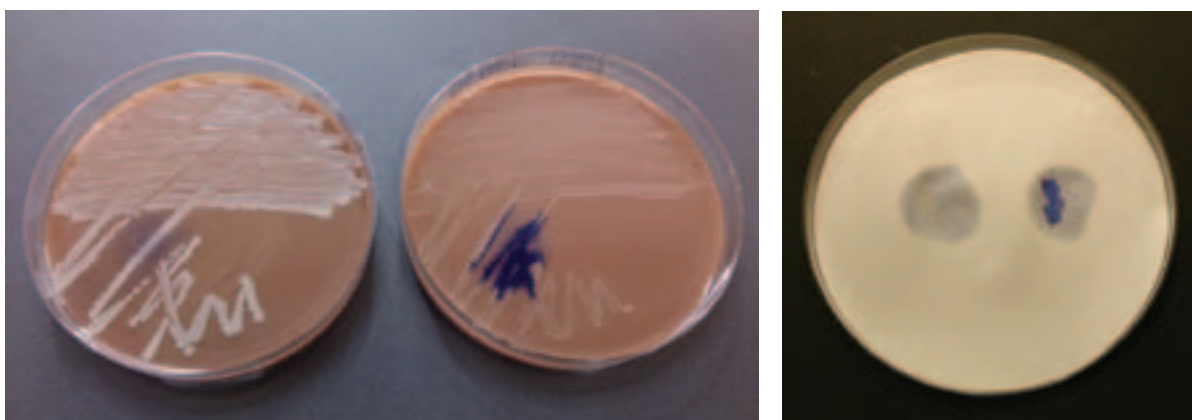
Figure 4.5
Oxidase-positive purple colonies of *N. gonorrhoeae* **on culture plate (left photo, right agar plate) and filter paper (right, which also shows a negative reaction that remains yellow), after reaction with 1% aqueous solution of tetramethyl-para-phenylenediamine dihydrochloride.**

4.5.5 Quality control (QC) of reagents for oxidase test

QC should be performed at regular intervals and always when using a new batch of reagents. Oxidase positive, such as *N. gonorrhoeae* (2008 WHO reference strain; *16*), and negative, such as *S. epidermidis* (ATCC 12228) or *E. coli* (ATCC 25922), reference strains can be used.

4.5.6 Confirmation of identification of *N. gonorrhoeae* after culture

Three approaches can be used for confirmation: use of biochemical tests that differentiate the divergent Neisseria and other closely related species and give a full species identification, use of immunological reagents, or use of molecular detection that are specific for *N. gonorrhoeae* but only confirm the identity of *N. gonorrhoeae* and do not speciate the isolates giving negative reactions. The choice among these approaches will be dependent on number of isolates to be tested, expertise, and cost. In laboratories that identify gonococcal isolates infrequently, it is advisable to use a commercially available kit that provides full identification. In laboratories that identify many isolates, *N. gonorrhoeae* is frequently confirmed using a specific reagent and then, if any are unexpectedly negative, these are further tested using a biochemical kit. No test is 100% sensitive and specific so, if resources are available, it is currently recommended that clinical isolates of *N. gonorrhoeae* should be confirmed using a combination of biochemical and immunological tests (or molecular tests where available). This approach is especially recommended for reference laboratories.

4.5.6.1 Tests that differentiate between Neisseria species

Traditionally, the ability of *N. gonorrhoeae* to produce acid from its utilization of glucose, detected by colour change of a pH indicator due to lowered pH, in comparison to, e.g. *N. meningitidis*, which additionally utilises maltose, has been the main method of identification (Table 4.4). This unique pattern of carbohydrate utilization is detected by inoculation of pure cultures into cysteine trypticase agar (CTA) containing glucose, maltose, and sucrose, respectively, at a final concentration of 1–2% following 24 hours incubation (Fig. 4.6; *17*).
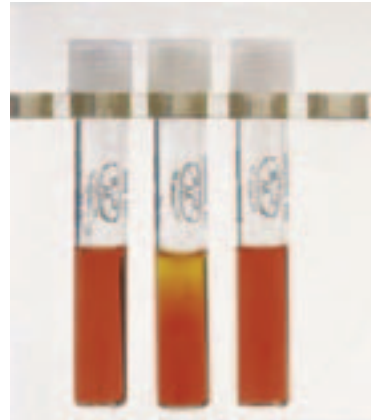
Figure 4.6
Acid production from carbohydrate utilization in cysteine trypticase agar (CTA) medium. Tubes from left to right are CTA base medium containing no carbohydrate, CTA medium containing 1% glucose, and CTA medium containing 1% maltose. The inoculated bacterial isolate is *N. gonorrhoeae*. Source: Reproduced from Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010.

It is essential to perform this test using a pure culture; this may necessitate at least one subculture resulting in a longer turnaround time for confirming the identification than more rapid tests described below.

The rapid carbohydrate utilization test (RCUT) (*18*, *19*), which is dependent on preformed enzymes and not on growth, using liquid media inoculated with a heavy pure growth, which produce results in 4 hours, is also effective.

The rapid detection of preformed enzymes also requires a pure culture (which should always be taken from non-selective medium), but does not require overnight incubation for the test and provides faster results. For all these tests, it is important to follow the instructions from the manufacturer precisely. Primarily, these tests detect different enzymes in the aminopeptidase pathway and include Gonochek-II (E-Y Laboratories) (*17*, *19*) and Neisseria Preformed Enzyme Test (PET; Key Scientific) (*17*). The Gonochek-II test is used to differentiate the Neisseria species via the detection of three preformed enzymes (prolyliminopeptidase [PIP], γ-glutamyl-aminopeptidase and β-galactosidase).

Table 4.4: Carbohydrate (sugar) utilization and enzyme activity of different Neisseria species and other oxidase-positive species such as *Moraxella catarrhalis* and *Kingella denitrificans*

Species	Biochemical activity							
	Glucose	Maltose	Lactose	Saccharose (Sucrose)	Fructose (Levulose)	ONPG[a]	GGT[b]	PIP[c]
N. gonorrhoeae	+	−	−	−	−	−	−	+ (−)
N. meningitidis	+	+	−	−	−	−	+ (−)	− (+)
N. lactamica[d]	+	+	+	−	−	+	−	+
N. polysaccharea[d]	+	+	−	+/−	−	−	−	+
N. cinerea[d]	− (+)	−	−	−	−	−	−	+
N. subflava[d, e]	+	+	−	+/−	+/−	−	−	+
N. sicca	+	+	−	+	+	−	−	+
N. mucosa	+	+	−	+	+	−	−	+
N. flavescens	−	−	−	−	−	−	−	+
M. catarrhalis	−	−	−	−	−	−	−	− (+)
K. denitrificans	+	−	−	−	−	−	−	+

[a] ONPG, o-nitrophenyl-β-galactosidase.

[b] GGT, γ-glutamyl aminopeptidase (transferase).

[c] PIP, prolyliminopeptidase (hydroxyproline aminopeptidase; HPA; proline arylamidase; PRO).

[d] *N. lactamica* usually grows on gonococcal selective medium, but also these other non-pathogenic saprophytic Neisseria species may appear.

[e] Includes biovars subflava, flava, and perflava, which differ in their activity against saccharose and fructose.

+/−, not consistent for the species; + (−), mostly positive but negative strains exist; − (+), mostly negative but positive strains exist.

A change to a blue colour indicates there is hydrolysis of 5-bromo-4-chloro-3-indolyl-β-galactoside by β-galactosidase, which is indicative of *N. lactamica*. A yellow colour indicates hydrolysis of γ-glutamyl-para-nitroanilide by γ-glutamyl aminopeptidase, which is a characteristic of *N. meningitidis*. In the absence of a colour change, the primary lid is removed and replaced by the secondary lid, which has a diazo dye (colour developer) incorporated and the tube inverted. If a red colour is observed, this indicates hydrolysis of L-proline 4-methoxynaphtylamide and the presence of PIP, giving a presumptive identification of *N. gonorrhoeae*. The Neisseria PET test works on a similar principle with a few variations. Following incubation at 37°C for 30 minutes, the colour change occurs as described for Gonochek-II. If there is no colour after 30 minutes, one drop of PET reagent is added and the colour recorded after a further 2 minutes.

It should be noted that some saprophytic Neisseria species, such as *N. cinerea*, *N. polysaccharea*, and *N. subflava*, may appear on specialized gonococcal media after 24–48 hours of incubation; these species possess PIP and, therefore, may produce misleading false-positive results with the Gonochek-II and Neisseria PET kits. False negatives also can occur with isolates of *N. gonorrhoeae* that lack an expression of a functional prolyliminopeptidase enzyme, and there has been worldwide dissemination of a PIP-negative gonococcal clone (*20*). Occasional isolates of *N. meningitidis* from urogenital sites lack γ-glutamyl aminopeptidase.

Species-verification using preformed enzyme tests alone is not recommended. However, a combination of carbohydrate utilization and detection of preformed enzymes, which is commercially available in rapid kits, gives a more reliable identification.

It is essential to follow strictly the instructions from the manufacturer for these tests. API-NH (bioMerieux) and RapID NH (Remel) (*17*) are two examples of such kits, which unfortunately may be too expensive for less-resourced settings. They both contain dehydrated substrates in a series of cupules or wells, which are filled with a suspension of the colonies to be identified; following 2–4 hours incubation at 37°C, colour reactions are recorded. A profile number is produced and the identification obtained by comparison with a database (paper or online). The API NH has 10 wells (allowing 13 identification tests), which include a β-lactamase test, 4 carbohydrate utilization tests, and 8 biochemical tests for different enzyme-substrate reactions (Fig. 4.7). The RapID NH consists of 2 carbohydrate cavities and 11 biochemical wells for different enzyme-substrate reactions (see the main reactions for distinguishing Neisseria species in Table 4.4).

4.5.6.2 Quality control (QC) of biochemical species-verifying assays

Each new batch of commercially available assays or in-house reagents should be quality controlled using reference strains of appropriate bacterial species such as *N. gonorrhoeae* (2008 WHO reference strain (*16*)), *N. meningitidis* (ATCC BAA-335), *N. lactamica* (ATCC 23970), *N. sicca* (ATCC 9913), and *N. cinerea* (ATCC 14685). Furthermore, every time the species confirmative assay is performed, the same reference strains should be included as controls.

Recently, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) has started to be introduced in well-resourced laboratories for species verification of most bacterial species including Neisseria species (*21*). The equipment for this sophisticated method is expensive. However, the cost per species-verified isolate is low and the method is easy to perform and very rapid. MALDI-TOF MS seems to distinguish effectively between commensal Neisseria species and pathogenic Neisseria species, as well as distinguish *N. meningitidis* from *N. gonorrhoeae*. However, additional evaluations are necessary. When using MALDI-TOF MS systems for species verification of *N. gonorrhoeae*, it is important to follow the instructions from the manufacturer precisely.

4.5.6.3 Tests specific for N. gonorrhoeae

Confirmation by tests that are specific for *N. gonorrhoeae* can be immunologically based or molecularly based and are particularly useful in laboratories that encounter large numbers of isolates for identification and can afford these relatively expensive tests. These assays may be performed directly with colonies on the selective isolation plate and do not require the use of a pure subculture. This means that an isolate can be identified at least 24 hours earlier than is possible with the rapid carbohydrate or enzyme substrate assays.

The most popular of commercially available kits is the Phadebact Monoclonal GC test (Boule) (*17, 19*), which contains a mixture of monoclonal antibodies directed at the major outer membrane porin PorB (Por). These antibodies are absorbed, via their Fc segment, onto protein A from *Staphylococcus aureus* and when mixed with gonococcal antigen, agglutination occurs. The

Figure 4.7
API NH identification kit demonstrating the profile of *N. gonorrhoeae*

test requires a light suspension of the test organism (approximately a density of 0.5 according to McFarland nephelometric standards; see Annex 4) made in the manufacturer's buffer or 0.9% sterile phosphate-buffered saline (PBS) solution, which is then boiled for 10 minutes and allowed to cool before use. The suspension should be boiled immediately after it has been prepared, as delay will result in lysis of the bacteria and release of bacterial DNA, which can cause auto-agglutination and difficulty in reading the test. One drop of the suspension is mixed for 2 minutes with each of two reagents, which allows for serogrouping into WI (IA; PorB1a) and WII/III (IB; PorB1b) in addition to identification (Fig. 4.8). The reagent is coloured blue to aid reading the agglutination reaction. This test has a high sensitivity and specificity but is a mixture of specific antibodies rather than a single antibody to a conserved antigen. False negatives are unusual but do occur.

The immunofluorescent reagent MicroTrak *N. gonorrhoeae* culture confirmation test (Trinity Biotech) (*17*, *19*) uses a similar pool of monoclonal antibodies to PorB but is linked to fluorescein. A thin smear of four or five colonies is emulsified in distilled water on a microscope slide, fixed, and covered with the reagent. After incubation at 37°C in a moist chamber, the slide is washed, allowed to dry, and then examined for the presence of fluorescent green diplococci under 100× oil objective using a fluorescence microscope. This test also was highly sensitive and specific and could be used directly on primary isolation media but has unfortunately recently been discontinued.
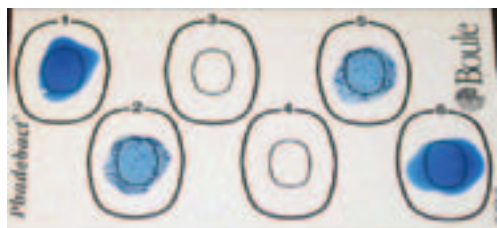
Figure 4.8
Reaction of two *N. gonorrhoeae* strains (A, wells 1 and 2, and B, wells 5 and 6) with Phadebact coagglutination reagents WI (wells 1 and 5) and WII/III (wells 2 and 6).
Source: Reproduced from Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010.

GonoGen II (*17*, *19*) also employs a similar panel of antibodies, but in this test the monoclonal antibodies are adsorbed onto suspended metal-sol particles. When the culture is emulsified in the solubilising buffer, the outer membrane of the organism is stripped off, releasing the PorB-containing complexes into solution. These released PorB complexes then are captured by the antibody/metal-sol particles. The sample/reagent mixture then is filtered through the special matrix device; the PorB antibody/metal-sol complexes are held back by the matrix, resulting in a red spot. Antibody/metal-sol particles that have not bound PorB will pass through the matrix giving a negative result (white to pale pink ring).

4.5.6.4 Quality control (QC) of immunological identification tests

Each new batch of commercially available assays or in-house reagents should be quality controlled using *N. gonorrhoeae* reference strains (positive controls for both serogroup WI (IA, PorB1a; e.g. 2008 WHO reference strain G; *16*) and WII/III (IB, PorB1b; e.g. 2008 WHO reference strain K; *16*) as well as a negative reference strain of other closely related species, e.g. *N. lactamica* (ATCC 23970). In addition, the identical reference strains should be included each time the method is performed.

Molecular confirmation of identity can be performed using the NAATs described in section 4.6, but this is probably most applicable for use in reference laboratories.

- Culture is sensitive and highly specific in optimized circumstances, is relatively inexpensive, and provides a viable organism for antimicrobial susceptibility testing.
- Due to the high level of antimicrobial resistance in gonococci worldwide, it is essential to keep and, in several settings, strengthen the culture capacity to allow surveillance of antimicrobial resistance.
- For sensitive and specific culture, sample collection, transportation, storage, and culture methodology need to be optimized and quality assured.

4.6 Molecular detection

4.6.1 Introduction and molecular assays

Molecular detection of specific nucleic acid (DNA/RNA) sequences of *N. gonorrhoeae* is most commonly performed using commercially available kits that detect both *N. gonorrhoeae* and *C. trachomatis*, in the same kit and sometimes simultaneously, and often at little or no extra direct cost. The first developed molecular tests were the non-amplified nucleic acid hybridization (NAH) assays such as PACE 2 (Gen-Probe) and Hybrid Capture 2 (HC2) CT/NG (Digene Corporation). NAH assays rely on the binding of specific complementary nucleic acid probes and subsequent signal amplification to detect binding. However, NAH assays are substantially less sensitive than NAATs, and should not be used diagnostically when NAATs are affordable.

Presently, NAATs are mainly used for detection of *N. gonorrhoeae*. The gonococcal NAATs detect a region of the DNA or rRNA specific to *N. gonorrhoeae*, and the region differs between kits (see Table 4.5). The target sequence is amplified using a variety of methods (Table 4.5) to produce multiple copies that can be easily detected. For basic information regarding NAH assays and different NAAT technologies, see Annex 3 and Chapter 5 (*C. trachomatis*). The gonococcal NAATs are highly sensitive and specific (but specificity differs substantially between the NAATs) and can be used with noninvasively taken specimens (e.g. urine in men and vaginal swabs in women). This allows both a greater number of patients to be seen in a clinic or primary care setting as well as providing the prerequisites for effective screening. NAATs can usually be performed within a working day, giving a faster turnaround time than culture (minimum of 2–3 days), and are often used in combination with robotics, enabling a high throughput. The sensitivity of NAATs is often quoted as higher than culture and this reflects their greater tolerance to inadequacies in the collection, transportation, and storage process. Disadvantages of using NAATs for detection of *N. gonorrhoeae* include the cost of equipment and reagents, current absence of a licensed commercial NAAT for extra-genital specimens, inability to perform antimicrobial susceptibility testing, and the suboptimal specificity of some NAAT assays

(see sections 4.6.2–4). Despite these disadvantages, the use of appropriate NAATs in addition to culture (for antimicrobial susceptibility testing) should be encouraged even in resource-constrained settings. This can be facilitated by the creation and support of regional reference laboratories that can provide diagnostic services using these methods. Regional reference laboratories offer many advantages as a result of larger testing volumes, rigorous adherence to good laboratory practices, and improved technical expertise. Use of regional laboratories may reduce costs while using assays with the highest sensitivity, and result in equivalent or reduced turnaround times.

- NAATs generally offer the highest sensitivity, especially for pharyngeal and rectal samples (despite not being licensed), are usually highly specific, and can be used with noninvasively taken samples.
- Molecular assays are more tolerant to inadequacies in collection, transportation, and storage conditions, and are objective.
- The specificity of several of the gonococcal NAATs is suboptimal (see sections 4.6.2–4), which results in low PPVs in low prevalence populations; supplementary NAATs that target another sequence may be required for confirmation.
- If internationally approved NAATs cannot be used, it is strongly recommended that the effectiveness of the proposed NAAT for the local settings, before use, is strictly validated and quality assured against at least one internationally approved NAAT.

New NAAT assays are becoming available rapidly and cannot be anticipated in this document. It is important to continually assess the relevant literature for high quality evaluations of new assays to determine the best fit for each laboratory.

4.6.2 Specimen types for *N. gonorrhoeae* NAATs

A range of different specimen types can be used with NAATs, including invasively taken specimens such as

Table 4.5: United States of America Food and Drug Administration (FDA)-approved NAATs for detection of *N. gonorrhoeae* (June 2012)

Test	APTIMA Combo 2 (AC2)	Cobas Amplicor	Cobas 4800	Probetec ET	Probetec GC Qx	Real Time CT/NG
Manufacturer	Gen-Probe	Roche	Roche	Becton, Dickinson	Becton, Dickinson	Abbott
Target	16S rRNA	Cytosine DNA methyl-transferase gene	Direct Repeat Region 9 (DR9)	PivNg (Pilin inverting protein homologue)	Pilin (different region from Probetec ET)	Opa genes
Technology	Transcription-mediated amplification (TMA)	Polymerase chain reaction (PCR)	Real-time PCR	Strand displacement amplification (SDA)	SDA	Real-time PCR
Supplementary test available	Yes (different region of 16S rRNA)	No	No	No	No	No

liquid Papanicolaou (Pap) specimens, cervical swabs, and urethral swabs (men); and noninvasively taken specimens such as vaginal swabs and urine (men and women). However, different manufacturers produce NAATs that are licensed for different specimen types, so it is essential to check the manufacturer's instructions or to conduct extensive in-house validation. It should be noted that urine is not the optimal sample, due to reduced sensitivity, for detection of *N. gonorrhoeae* in women (*22*). It is essential to follow the instructions from the manufacturer precisely regarding approved samples; collection, transportation, and storage of samples; and performance of NAAT.

There are no licensed NAAT for use with extra-genital samples, rectal and pharyngeal, but there is increasing evidence that NAATs are more sensitive than culture at these sites (*4–6*). Validation data exists to support their use, but it is recommended that a positive test at either of these sites should be confirmed using a supplementary test (NAAT with another target sequence) to avoid false-positive results (*23, 24*). The choice of NAAT for pharyngeal specimens should be made with care to avoid those known to show cross-reactivity with commensal Neisseria species or *N. meningitidis*.

4.6.3 Specificity of *N. gonorrhoeae* NAATs

Historically, there has been concern over the specificity of the target chosen, as the species in the Neisseria genus are genetically closely related and a large number of the commensal Neisseria species are found in the pharynx, rectum, and sometimes in the lower genital tract. Identification of a target sequence specific to *N. gonorrhoeae* has been a challenge, and cross-reactivity has been reported with many non-gonococcal Neisseria species for some kits, including *N. meningitidis*, *N. cinerea*, *N. flavescens*, *N. lactamica*, *N. sicca*, and *N. subflava* (*22–28).* The most recent generation of kits currently on the market has improved markedly in this regard, but it still remains a factor to be considered when selecting the appropriate kit for the specimen type to be tested as not all NAATs for gonorrhoea are equal in this regard (*26, 28*).

4.6.4 Sensitivity, specificity, and prevalence: the effect on positive predictive value (PPV)

The prevalence of gonorrhoea in the population being tested must be considered together with the sensitivity and specificity of the NAAT being used as this will affect the PPV of the test and hence the number of false

positives obtained. This is demonstrated in Table 4.6. A PPV of >90% (using single NAAT or screening NAAT plus supplementary NAAT with different target) has been suggested as a minimum when using NAATs to detect *N. gonorrhoeae*.

It should be noted that, even when the sensitivity and specificity of a NAAT is above 95%, the PPV in a population of both 1% and 5% is for most NAATs still less than 90% whereas, at a prevalence of 10%, the PPV of most (but not all) NAATs is greater than 90%. This is particularly important for dual NAATs that detect both *N. gonorrhoeae* and *C. trachomatis* because the prevalence of these two infections can differ markedly. In many countries, chlamydial infection is commonly detected at a considerably higher prevalence than gonorrhoea, and the algorithm for testing for both infections together may not need a supplementary test for detection of *C. trachomatis*, but will require the positives for gonorrhoea to be tested with a second NAAT (another target sequence) to obtain acceptable PPVs.

4.6.5 NAATs not approved by the United States of America Food and Drug Administration (FDA)

In-house NAATs that target, for example, the *cppB* gene, cytosine DNA methyltransferase (CMT) gene, *opa* genes, and *porA* pseudogene have been described (*24*, *29–32*) and are used extensively in some countries. The most successful are those that target the *porA* pseudogene and *opa* genes either separately or in combination. The *porA* pseudogene is absent in commensal Neisseria species (and the *porA* gene in *N. meningitidis* is sufficiently different), and consequently this target has proven highly specific for *N. gonorrhoeae*. Nevertheless, several countries have reported recently rare *N. gonorrhoeae* isolates containing a meningococcal *porA* instead of a gonococcal *porA* pseudogene, resulting in false-negative NAAT results (*33–36*). NAATs that target the *cppB* gene can vary in sensitivity and specificity, as some isolates of *N. gonorrhoeae* do not carry this gene and conversely some strains of *N. meningitidis* do have this gene and cross-react. Furthermore, the NAATs targeting the CMT gene can cross-react with commensal Neisseria species. Hence, the NAATs targeting the *cppB* gene and CMT gene are not recommended. The in-house

Table 4.6: Effect of prevalence on positive predictive value (PPV) for single tests

Tests		A	B	C
Sensitivity		97.8%	96.4%	98.0%
Specificity		99.2%	97.9%	99.7%
PPV	10% prevalence	93%	84%	97%
	5% prevalence	87%	73%	95%
	1% prevalence	55%	35%	77%

NAATs may require more technical expertise; however, the sensitivity and specificity of these tests can be high and they can be a less expensive and effective option, particularly for small numbers of samples. Multiplex polymerase chain reaction assays detecting, e.g. *N. gonorrhoeae*, *C. trachomatis*, *Mycoplasma genitalium*, and *Trichomonasis vaginalis* also have been developed; however, these need further evaluation regarding their performance characteristics.

Accordingly, globally there are many commercially available or even in-house developed *N. gonorrhoeae* NAATs in use. If any NAATs that have not been approved by the FDA are used, regional, such as European Union, and/or other national regulatory processes should provide safeguards on the quality and performance of the diagnostic NAAT. It is strongly recommended that only internationally approved NAATs are used. If this is not possible, it is essential that the proposed NAAT, before use, is strictly validated for local requirements against at least one internationally approved NAAT and subsequently used with appropriate positive, negative, and inhibition controls, as well as participation in an appropriate external quality assessment (EQA) system.

4.6.6 Quality control (QC) and quality assurance (QA) of NAATs

Internal quality controls (IQC) should be included in each test run. Internal quality assessment (IQA) should be performed regularly by retesting samples to which the original result has been blinded. The number and frequency of these IQAs will be dependent on the total number of tests performed; an example is 1–5% of the total tested each month. EQA can be achieved by using

panels of specimens from appropriate EQA providers, such as United Kingdom National External Quality Assessment Services (UK NEQAS; www.ukneqas.org.uk), or Quality Control of Molecular Diagnostics (QCMD; www.qcmd.org) which deliver to many countries, or by a more informal exchange of samples between laboratories.

4.7 Point-of-care (POC) tests ("rapid tests")

No POC test ("rapid test") for antigen detection with appropriate performance characteristics, e.g. sensitivity and specificity, is available; therefore, none can be recommended for the diagnosis of uncomplicated or complicated gonorrhoea. However, some new POC tests utilizing latest technology are under development and, with appropriate performance characteristics, these would be exceedingly valuable for rapid diagnosis at site, e.g. at the clinic or in the field. Especially in resource-constrained, high-prevalence populations, decreases in sensitivity may be acceptable in exchange for the ability to test and treat while the patient is on-site (*37, 38*). The POC tests could in these settings also be used to increase the specificity of the syndromic management algorithms, which will reduce overtreatment, and find many asymptomatic infections, especially in women. For diagnosis of gonorrhoea in men, microscopy after Gram staining is a type of POC test; however, this method lacks adequate sensitivity in women.

- No rapid POC test with appropriate sensitivity is available; therefore, none can be recommended for the diagnosis of gonorrhoea.
- However, some new POC tests utilizing the latest technology are under development and, with appropriate performance characteristics, these would be exceedingly valuable for rapid diagnosis and immediate treatment of the patient.

4.8 Antimicrobial susceptibility testing

4.8.1 Introduction

N. gonorrhoeae has developed resistance to all previous first-line antimicrobials for treatment of gonorrhoea, e.g. penicillins, tetracycline, and fluoroquinolones, leaving the expanded-spectrum cephalosporins ceftriaxone and cefixime as the only antibiotics recommended for treatment of gonococcal infections in many countries (*2*). During the past decade, susceptibility to the expanded-spectrum cephalosporins has also decreased in many regions worldwide, and treatment failures with cefixime have been verified in several countries (*39–44*). Recently, the first extensively-drug resistant (XDR; *2*) gonococcal strains with high-level resistance also to ceftriaxone (the last remaining option for empirical first-line treatment) were verified (*3, 43*). If ceftriaxone-resistant strains spread globally, gonorrhoea will become untreatable with single-antimicrobial regimens in certain circumstances, and especially in some settings (*2*). Accordingly, it is crucial to monitor the antimicrobial susceptibility of *N. gonorrhoeae* locally, regionally, and globally. WHO has revisited and revamped the WHO Global Gonococcal Antimicrobial Susceptibility Surveillance Programme (GASP). For further information about essential antimicrobial resistance surveillance worldwide and WHO Global GASP, see the WHO Surveillance Standards for Gonococcal Antimicrobial Resistance, Appendix 4. In June 2012, WHO also launched the *WHO global action plan to control the spread and impact of antimicrobial resistance in* Neisseria gonorrhoeae (available at: www.who.int/reproductivehealth/publications/rtis/9789241503501/en/).

The agar dilution method is the recommended "gold standard" method for antimicrobial susceptibility testing or determination of the minimum inhibitory concentration (MIC; in µg/ml or mg/l) of gonococcal isolates to antimicrobial drugs. However, this method can be laborious and less suited for routine susceptibility testing, especially if testing a low number of strains. Therefore, the standardized and quality-assured Etest method, which correlates closely with the agar dilution method, is commonly used. A qualitative determination of antimicrobial susceptibility can be obtained using disc diffusion assay. Several disc diffusion methods are also in use; however, these require pronounced standardization and appropriate QC to attain a high level of reproducibility and correct interpretation to reflect adequately the MIC values of the different antimicrobials. The disc diffusion methods are inexpensive but only recommended for use when MIC determination cannot

be performed, due to limited resources or other reasons. If using a disc diffusion method, it is recommended that the finding of any new, emerging, or rare antimicrobial resistance is confirmed by MIC determination. β-lactamase production is often determined by a chromogenic cephalosporin test, using nitrocefin discs or nitrocefin solution.

All methods for antimicrobial susceptibility testing should be performed from pure, fresh (18–24 hours) *N. gonorrhoeae* cultures taken from non-selective culture media. The isolates also should have been appropriately species-verified and subcultured at least once. In antimicrobial susceptibility testing, it is important to follow all steps of the nominated method precisely, including selection and use of agar medium, reagents (antimicrobial powder, Etest strips, discs, and buffers), inoculation, incubation, and interpretation.

- Due to the high level of antimicrobial resistance in gonococci worldwide and fear that gonorrhoea may become untreatable in certain circumstances and especially in some settings, it is essential to monitor the antimicrobial susceptibility of *N. gonorrhoeae*.
- MIC determination is performed by agar dilution method or Etest.
- A qualitative determination of antimicrobial susceptibility can be obtained using disc diffusion methods.
- Disc diffusion methods require standardization and appropriate QC and, importantly, do not measure and only reflect the MIC. These methods should only be used when MIC determination cannot be performed, e.g. due to resource constraints.
- In all antimicrobial susceptibility testing, it is essential to follow the nominated method precisely, which should be appropriately standardized, validated, and quality-assured.

4.8.2 Choice of antimicrobials included in antimicrobial susceptibility testing

The list of antimicrobials to be tested should include drugs nationally or regionally recommended and used for treatment of gonococcal infections as well as drugs recommended by the local antimicrobial susceptibility surveillance programme. However, especially at reference laboratories, additional antimicrobials can be tested, such as antimicrobials recommended for treatment in other settings, antimicrobials that may be candidates for future treatment, and drugs useful for local longitudinal studies of *N. gonorrhoeae*.

4.8.3 Determination of MIC (agar dilution and Etest)

4.8.3.1 Recommended agar medium

The medium recommended for determination of MIC of different antimicrobials, using agar dilution or Etest, for *N. gonorrhoeae* isolates is an appropriate GC agar base, such as Difco GC Medium Base, supplemented with 1% defined growth supplement* or 1% IsovitaleX/Vitox. As an example, see the GCVIT medium described in Annex 4.

*In accordance with the Clinical and Laboratory Standards Institute (CLSI; *45*): 1.1 g L-cysteine, 0.03 g guanine HCl, 3 mg thiamine HCl, 13 mg para-aminobenzoic acid (PABA), 0.01 g B_{12}, 0.1 g cocarboxylase, 0.25 g NAD, 1 g adenine, 10 g L-glutamine, 100 g glucose, and 0.02 g ferric nitrate (in 1 l H_2O). Cysteine-free growth supplement is required for agar dilution tests with carbapenems and clavulanate.

4.8.3.2 Interpretative criteria

Interpretative criteria for susceptibility, intermediate (decreased) susceptibility, and resistance that are recommended using MIC determination are described in Table 4.7. These criteria are from CLSI (*45*), with exception of the criteria for azithromycin (for which CLSI (*45*) has not stated any criteria) that are from the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org). EUCAST is another organization that states interpretative criteria for antimicrobial susceptibility testing, for *N. gonorrhoeae*; in general, their breakpoints are slightly lower than the ones recommended by CLSI.

4.8.3.3 Quality control (QC) of MIC determination (agar dilution and Etest)

An appropriate selection of the 2008 WHO *N. gonorrhoeae* reference strains (*16*) ideally should

Table 4.7: MIC interpretative criteria for categorizing *N. gonorrhoeae* into susceptibility categories according to CLSI, with exception of azithromycin from EUCAST

Antimicrobial	MIC (mg/l)		
	Susceptible (S)	Intermediate susceptible (I)	Resistant (R)
Ceftriaxone	≤0.25	TBD[a]	TBD[a]
Cefotaxime	≤0.5	TBD[a]	TBD[a]
Cefixime	≤0.25	TBD[a]	TBD[a]
Cefpodoxime	≤0.5	TBD[a]	TBD[a]
Benzylpenicillin	≤0.06	0.12–1	≥2
Ciprofloxacin	≤0.06	0.12–0.5	≥1
Ofloxacin	≤0.25	0.5–1	≥2
Spectinomycin	≤32	64	≥128
Azithromycin[b]	≤0.25	0.5	≥1[c]

MIC, minimal inhibitory concentration.

[a] TBD, to be determined. Due to the lack of adequate number of resistant strains and evidence-based correlates between the MIC of isolates and treatment outcome, the breakpoints cannot be determined yet.

[b] CLSI (*45*) has not stated any breakpoints for azithromycin and, accordingly, the breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) are given.

[c] In North and South America, a resistance breakpoint of ≥2 mg/l is frequently used, which has been recommended by the United States of America Centers for Disease Control and Prevention (www.cdc.gov/std/GISP2007/).

be included with each batch of susceptibility testing, and always when a new batch of antimicrobial powder, agar medium, or Etest strips are used. The MIC of each antimicrobial and reference strain should be documented on a QC chart. For acceptable MIC values of different antimicrobials in the QC, see Table 4.8. The 2008 WHO *N. gonorrhoeae* reference strains (*16*) are also available at the National Collection of Type Cultures, United Kingdom (NCTC; www.hpacultures.org.uk/collections/nctc.jsp), named as NCTC 13477–13484; and at the Culture Collection University of Gothenburg, Sweden (CCUG; www.ccug.se), named as CCUG 57595–57602. The 2008 WHO *N. gonorrhoeae* reference strains (*16*) also can be used for QC in other phenotypic and genetic laboratory tests for *N. gonorrhoeae* as well as in EQA systems. The WHO Surveillance Standards for Gonococcal Antimicrobial Resistance, Appendix 4 describes in detail the applications for the 2008 WHO *N. gonorrhoeae* reference strains (*16*) and instructions for their use. However, all eight 2008 WHO reference *N. gonorrhoeae* strains (*16*) do not have to be used for QC

in the antimicrobial susceptibility testing. Many settings mainly utilize WHO G, WHO K, WHO M, WHO O, and WHO P. The 2008 WHO *N. gonorrhoeae* panel, the original panel (strains A–E), and additional WHO *N. gonorrhoeae* reference strains are also available from WHO sources, such as the WHO Collaborating Centre for HIV and STDs, Sydney, Australia, and the WHO Collaborating Centre for Gonorrhoea and other STIs, Örebro, Sweden.

4.8.4 Agar dilution method for MIC determination

4.8.4.1 Introduction

Agar dilution method is the recommended "gold standard" method for quantitative antimicrobial susceptibility testing or determination of the MIC of gonococcal isolates to antimicrobial drugs. Antimicrobial agents are incorporated into GC agar base supplemented with 1% defined growth supplement or 1% IsovitaleX/Vitox (see section 4.8.3.1) in serial twofold dilutions. *N. gonorrhoeae* isolates to be tested are grown overnight

Table 4.8: Acceptable MIC ranges and antimicrobial susceptibility categorization (modified from reference *16*), and some additional typing characteristics for the 2008 WHO *N. gonorrhoeae* reference strains recommended for QC. Acceptable MIC ranges for *N. gonorrhoeae* ATCC 49226 recommended by CLSI are also given.

Antimicrobial agent (mg/L)	WHO reference strains								ATCC 49226[a]
	WHO F	WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	
Penicillin G	0.016–0.064 (S)	0.25–1.0 (I)	1.0–4.0 (R)	1.0–4.0 (R)	4.0–16.0 (R)	4.0–16.0 (R)	>32 (R)	0.125–0.5 (I)	0.25–1.0
Cefixime	<0.016 (S)	<0.016 (S)	0.25–1.0 (DS)[b]	0.125–0.5 (DS)[b]	<0.016 (S)	<0.016 (S)	<0.016–0.032 (S)	<0.016 (S)	0.004–0.032
Ceftriaxone	<0.002 (S)	0.004–0.016 (S)	0.032–0.125 (DS)[b]	0.064–0.25 (DS)[b]	0.008–0.032 (S)	0.002–0.008 (S)	0.016–0.064 (S)	0.002–0.008 (S)	0.004–0.016
Erythromycin	0.25–1.0 (S)	0.5–2.0 (I)	0.5–2.0 (I)	1.0–4.0 (I)	0.5–2.0 (I)	0.25–1.0 (S)	0.5–2.0 (I)	2.0–8.0 (R)	1.0–2.0[c]
Azithromycin	0.064–0.25 (S)	0.125–0.5 (S)	0.125–0.5 (S)	0.25–1.0 (I)	0.125–0.5 (S)	0.064–0.25 (S)	0.125–0.5 (S)	1.0–4.0 (R)	0.5–1.0[c]
Ciprofloxacin	0.002–0.008 (S)	0.064–0.25 (I)	>32 (R)	>32 (R)	1.0–4.0 (R)	2.0–8.0 (R)	0.004–0.016 (S)	0.002–0.008 (S)	0.001–0.008
Spectinomycin	16–64 (S)	8–32 (S)	8–32 (S)	8–32 (S)	8–32 (S)	8–32 (S)	>1024 (R)	8–32 (S)	8.0–32.0
β-lactamase production	No	No	No	No	Yes	Yes	Yes	No	No
Serovar	Arst	Arst	Bpyust	Brpyust	Bpyust	Arst	Boys	Bopt	
NG-MAST[d]	ST3303	ST621	ST1424	ST1422	ST3304	ST556	ST495	ST3305	
PIP-negative[e]	No	Yes	No	No	No	Yes	No	No	

Note that the exact MICs shown should be used and interpreted with caution because these were derived using one specific Etest method only and, accordingly, may differ using other methods. However, the identified resistance phenotypes (SIR categorization) should be consistent between different methods.

[a] Acceptable ranges of MICs (CLSI; *45*).

[b] DS, decreased susceptibility because isolates with these MICs have the primary determinants for resistance to expanded-spectrum cephalosporins.

[c] Range established by National Microbiology Laboratory, Canada.

[d] *N. gonorrhoeae* multi-antigen sequence typing.

[e] Do not produce the enzyme prolyliminopeptidase (PIP), which can result in doubtful and/or false-negative species identification of *N. gonorrhoeae* using some biochemical or enzyme-substrate tests (*17*, *20*).

on non-selective GC agar medium and then suspended in Mueller–Hinton (MH) broth or sterile saline solution (or equivalent). Approximately 10[4] colony-forming units (CFU) then are inoculated onto the surface of the antimicrobial-containing media and two plates of antimicrobial-free control medium with a Steer's replicator, multipoint inoculator, or a calibrated loop. Plates are finally incubated overnight and subsequently examined for growth. The MIC of the antimicrobial agent for an isolate is the lowest concentration that inhibits its growth. A modification of the full MIC agar dilution method is the agar dilution breakpoint technique, which

is a similar method but with agar medium containing only one or two concentrations of the antimicrobials, which can be used to categorize isolates as being resistant (using an agar plate with concentration exactly at breakpoint for resistance) or having intermediate susceptibility (using an agar plate with concentration exactly at breakpoint for intermediate susceptibility). The breakpoint technique is useful for screening a large number of isolates.

4.8.4.2 Preparation of antimicrobial solutions

Appropriate antimicrobial powders or tablets to dissolve should be obtained directly from pharmaceutical companies or other validated providers. Because most antimicrobials are not 100% pure, the concentration incorporated in the agar plates should be based on the activity or potency (active drug per mg) of the antimicrobial, as specified by the manufacturer. The instructions from the manufacturer regarding dissolving the antimicrobial powder, expiry date, and storage instructions must be followed in detail. Antimicrobials should be incorporated into the agar medium in twofold dilutions, for example, using a scheme in which 1 part of antimicrobial solution is added to 9 parts of agar (see section 4.8.4.3).

For example, prepare stock solutions by dissolving 128 mg or a weight equivalent to 128 mg of active ingredient in a minimal amount of appropriate solvent (usually 5–10 ml, follow the instructions from the manufacturer precisely), and dilute further with distilled water (or other recommended solvent) to an exact volume of 25 ml. These stock solutions contain antimicrobial product at a concentration of 5120 mg/l, which is practical for preparing the working solutions. If not otherwise recommended by the manufacturer, the solvents given in Table 4.9 can be used in preparing stock solutions. Stock solutions can be sterilized by membrane filtration (0.22 μm filter), and stored in aliquots in tightly sealed vials at –20°C or, preferably, at –70°C for up to 6 months. When thawed, the solutions have to be used immediately and they should not be refrozen for later use.

For further use, prepare a series of twofold working dilutions for each antimicrobial containing antimicrobial concentrations 10 times higher than the final

concentrations to be obtained in the agar. An example of a standardized scheme for preparing the working dilutions is shown in Table 4.10.

The range of concentrations used in the antimicrobial susceptibility testing should be adapted to each antimicrobial and to local resistance levels. For many antimicrobials, wide variations exist in the susceptibility patterns of gonococcal isolates from different countries. To reduce the number of concentrations that have to be tested, an approximation of the local susceptibility variation for examined antimicrobials is required. If unknown, it may be obtained by determining the lower and upper limits of the susceptibility ranges for these antimicrobials on a small number of isolates. In general, it is more relevant and important to know the upper rather than the lower limits of the MIC values.

4.8.4.3 Preparation of plates for agar dilution method

For each antimicrobial and antimicrobial concentration to be tested, prepare a volume of, e.g. 89 ml appropriate GC agar base, such as Difco GC Medium Base (3.6 g GC

Table 4.9: Solvents for preparation of antimicrobial solutions for MIC determination using agar dilution method[a]

Antimicrobial	Solvent
Ceftriaxone	Distilled water
Cefixime	0.1 mol/l phosphate buffer, pH 7.0
Benzylpenicillin	Distilled water
Ciprofloxacin	Water or 0.1 mol/l HCl
Azithromycin	95% ethanol or glacial acetic acid[b]
Erythromycin	95% ethanol or glacial acetic acid[b]
Spectinomycin	Distilled water
Gentamicin	Distilled water
Kanamycin	Distilled water
Tetracycline	Distilled water
Chloramphenicol	95% ethanol

[a] If solvent and procedure for dissolvement of the antimicrobial powder is given by the manufacturer, follow the instructions from the manufacturer precisely.

[b] For glacial acetic acid, use one half volume of distilled water, then add glacial acetic acid dropwise until dissolved, not to exceed 2.5 μl/ml.

agar base and 89 ml distilled water) in a glass bottle, which is for preparation of four plates. Autoclave the agar medium in the bottle, then allow it to cool down to a temperature of 50°C in a water-bath before aseptically adding sterile supplements (1 ml enrichment/IsoVitalex/ Vitox) and 10 ml of antimicrobial working solution, i.e. in concentration to be tested (see Table 4.10). Immediately mix gently by inverting the bottle 3 times, remove the bottle top, flame the mouth of the bottle, and pour approximately 20–25 ml of medium into plates (diameter 90 mm) to give a layer of approximately 3.5–4.5 mm. Make sure to eliminate bubbles by gently swirling the plates or quickly flaming of the agar surface, e.g. with a Bunsen flame. Once the agar has solidified at

room temperature, store the plates inverted in sealed plastic bags at 4°C until use. In these conditions, there is no significant loss of antimicrobial activity for up to 2 weeks. However, for penicillin, which is less stable, it is recommended that the plates are used within 1 week. Agar plates without antimicrobials incorporated to be used as negative control should also be prepared.

4.8.4.4 Procedure of MIC determination using agar dilution

Preparation of bacterial inoculum: Use a sterile loop or swab to collect *N. gonorrhoeae* from an 18–24 hours pure culture on non-selective gonococcal agar medium and prepare a homogenized suspension of cells

Table 4.10: Preparing dilutions of antimicrobial agents for MIC determination using agar dilution method

| Step	Antimicrobial solution						Concentration of working dilution (mg/l)	Final concentration at 1:10 dilution in agar (mg/l)
	Concentration (mg/l)	Source	Volume (ml)	+	Volume distilled water (ml)	=		
1	5120	Stock	1					
2	5120	Stock	1		1		2560	256
3	5120	Stock	1		3		1280	128
4	5120	Stock	1		7		640	64
5	640	Step 4	1		1		320	32
6	640	Step 4	1		3		160	16
7	640	Step 4	1		7		80	8
8	80	Step 7	1		1		40	4
9	80	Step 7	1		3		20	2
10	80	Step 7	1		7		10	1
11	10	Step 10	1		1		5	0.5
12	10	Step 10	1		3		2.5	0.25
13	10	Step 10	1		7		1.25	0.125
14	1.25	Step 13	1		1		0.625	0.06
15	1.25	Step 13	1		3		0.3125	0.03
16	1.25	Step 13	1		7		0.156	0.016
17	0.156	Step 16	1		1		0.08	0.008
18	0.156	Step 16	1		3		0.04	0.004
19	0.156	Step 16	1		7		0.02	0.002
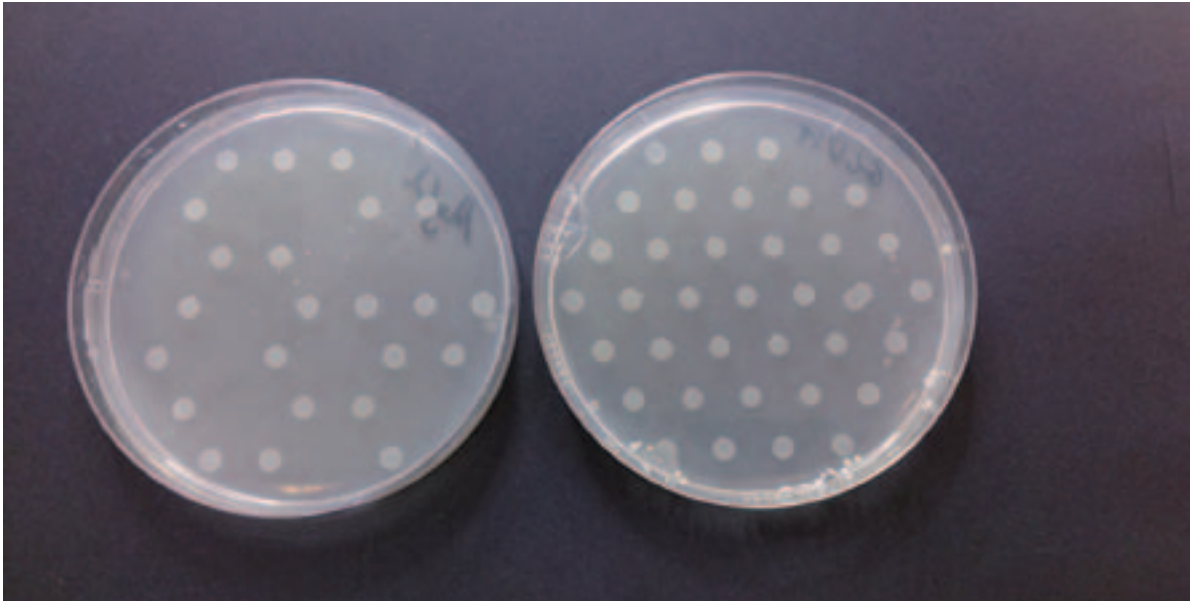
Figure 4.9
Results of MIC determination for *N. gonorrhoeae* using the agar dilution method. Medium containing 0.5 mg/l penicillin (left plate) shows inhibition of several gonococcal strains, while all strains grow well on the control medium (right plate).

(equivalent to 0.5 McFarland nephelometric standard, approximately 10^8 CFU per ml) in 1 ml of MH broth or sterile saline solution (suspension must be used within 15 minutes). Dilute the suspension 1:10 in MH broth or sterile saline solution to obtain 10^7 CFU/ml. Carefully transfer 0.5 ml of each suspension into the corresponding replicator or multipoint inoculator well.

Inoculation of plates: The agar plates must be dried before inoculation, i.e. by placing them in an incubator in an inverted position with the lids ajar. The replicator or multipoint inoculator should transfer approximately 1–2 μl of each suspension on to the agar surface in circular areas with diameters of 5–7 mm, giving a final bacterial inoculum of approximately 10^4 CFU per spot. If the replicator or multipoint inoculator transfers a smaller or larger volume of suspension, the dilution of the inoculum has to be adjusted, i.e. to give the final inoculum of approximately 10^4 CFU per spot. When testing small numbers of isolates, a sterile 1 μl plastic loop may be used. Inoculate a negative control plate (containing no antimicrobial) first, followed by the series of plates containing the different concentrations of antimicrobial, starting with the lowest concentration for each antibiotic.

Finally, inoculate a second negative control plate to ensure that there has been no contamination during the inoculation. The 2008 WHO *N. gonorrhoeae* reference strains (*16*) are recommended as QC (see Table 4.8). Allow the inocula to dry and incubate the plates inverted for 20–24 hours at 36±1°C in a 5±1% CO_2-enriched atmosphere with high humidity (70–80%). Sterilize the pin heads and wells by wrapping in foil and autoclaving, by placing them in a hot oven at 160°C for 2 hours, or by dipping them in 70% ethanol followed by flaming.

Reading of results: The results of the 2008 WHO reference strains (see Table 4.8 for acceptable MICs of different antimicrobials) and the negative control plates (should have pure and confluent gonococcal growth on both plates) must be reviewed and approved before reading other results. If not approved, perform trouble-shooting and repeat the testing. For tested isolates, record the MIC as the lowest concentration of antimicrobial agent that completely inhibits growth (Fig. 4.9).

Interpretation of results: Interpret results for tested isolates into category of susceptibility (see Table 4.7).

### 4.8.5 Etest method for determination of MIC

4.8.5.1 Introduction

Etest is a quantitative technique for determining the MIC of antimicrobial agents against microorganisms. Etest utilizes plastic strips calibrated with a MIC scale in µg/ml (mg/l) and a code to identify the antimicrobial agent. A predefined concentration gradient of antimicrobial is immobilized on the other surface of the strip. Once applied on to the surface of an agar plate, the antimicrobial diffuses into the medium. *N. gonorrhoeae* inoculated onto the medium (prior to addition of the strip) will show an elliptical zone of inhibition of growth after overnight incubation, if susceptible to the test antimicrobial. The MIC should be read as the intersection of the ellipse and the gradient scale marked on the strip. With regard to storage of Etest strips, performance of Etest, and reading of Etest results, follow the instructions from the manufacturer precisely.

4.8.5.2 Procedure of MIC determination using Etest

1. Dry (should be free of visible moisture, but do not over-dry them) the number of GCVIT agar plates (see Annex 4) needed. Allow the Etest strips to reach room temperature for approximately 30 minutes. An opened Etest package should be stored in an air-tight container with desiccant.

2. Allow the Etest strips to reach room temperature for approximately 30 minutes. An opened Etest package should be stored in an air-tight container with desiccant.

3. Use a sterile loop or swab to collect *N. gonorrhoeae* from an 18–24 hours pure culture on non-selective gonococcal agar medium and prepare a homogenized suspension of cells (0.5 McFarland nephelometric standard, approximately 10^8 CFU per ml) in 1 ml of sterile saline solution or PBS (suspension must be used within 15 minutes). Do not use a nutrient broth to prepare the suspension.

4. Dip a fresh sterile swab into the suspension and remove excess fluid by pressing and rotating the swab against the tube wall.

5. Swab the entire agar surface of the GCVIT plate evenly in three directions (Fig. 4.10) to produce a confluent lawn.

6. Replace the lid of the plate and allow the agar surface to dry for approximately 10 minutes.

7. Press the Etest applicator onto an Etest strip to pick it up (or use a sterile forceps), place it on the agar surface, and push the piston down to release the strip.

8. Confirm that the strip is in complete contact with the agar and remove possible air pockets carefully by streaking with a loop from lower to higher concentration of antimicrobial.

9. Place a maximum of four Etest strips per 140–150 mm plate and one strip per 90 mm plate.

10. Once applied to the agar, the Etest strip should not be removed (the antimicrobial is immediately released).

11. Immediately incubate inverted plates for 20–24 hours at 36±1°C in 5±1% CO_2 enriched humid (70–80%) atmosphere (CO_2 incubator or, if not available, candle extinction jar with some additional humidity).
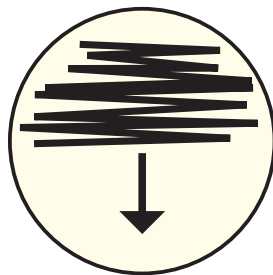
1. Swab down in one direction over the whole agar surface.
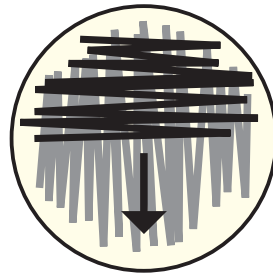
2. Turn the plate 90° and swab down in one direction over the whole agar surface.
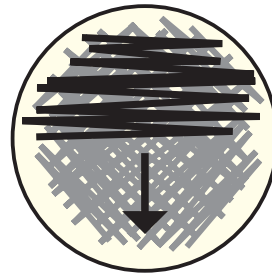
3. Turn the plate 45° and again swab down in one direction over the whole agar surface.

Figure 4.10
Swabbing of culture plates for Etest

4.8.5.3 *Reading and interpretation of Etest results after incubation*

1. The results of the 2008 WHO reference strains (see Table 4.8 for acceptable MICs of different antimicrobials), when included as QC, must be reviewed and approved before reading other results. If not approved, perform troubleshooting and repeat the testing. For tested isolates, read the plates only when sufficient growth is seen and the inhibition ellipse is clearly visible. Otherwise, the testing has to be repeated with more growth.

2. Read the exact MIC where the ellipse (zone) intersects the MIC scale on the Etest strip (Fig. 4.11). If the ellipse of inhibition intersects the Etest strip in-between two MIC values, always read the highest value. Always read the end point at complete inhibition of all growth, including hazes, microcolonies, and isolated macrocolonies.

3. Growth along the entire strip implies no inhibition, i.e. MIC is higher than (">") the highest concentration on the strip. If the inhibition ellipse is below the strip and does not intersect the strip, the MIC is lower than ("<") the lowest concentration on the strip.

4. Interpret results for tested isolates into category of susceptibility (see Table 4.7).
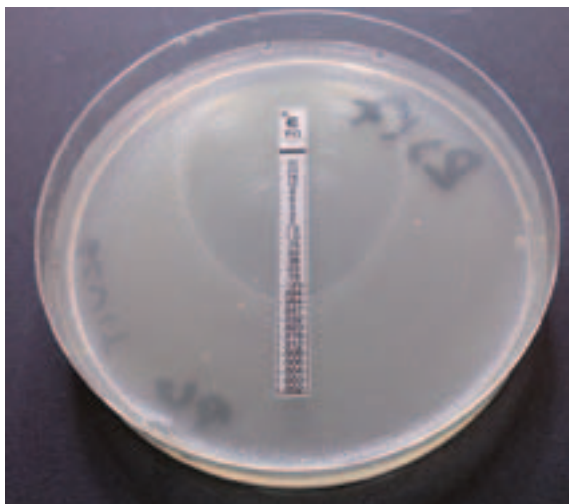
Figure 4.11
Results of MIC determination in *N. gonorrhoeae* using the Etest. The MIC of penicillin G (PG) is determined at the point the ellipse crosses the strip (0.094 mg/l).

4.8.6 Disc diffusion method

4.8.6.1 *Introduction*

Disc diffusion assays are qualitative techniques for categorizing isolates as susceptible, intermediate (or decreased) susceptible, or resistant to different antimicrobials. Accordingly, these methods do not determine the exact MIC of antimicrobials against microorganisms; however, they should reflect the MICs. The disc diffusion methods use commercially available discs that are impregnated with a known concentration of antimicrobial. The antimicrobial agent in the disc diffuses into the surface of the agar inoculated with the bacterial isolate and produces a concentration gradient that is highest close to the disc and proportionally decreases with the distance from the disc. After incubation, a zone of inhibition is visible that is measured and subsequently interpreted into a susceptibility category. Several disc diffusion methods are in use internationally; however, all require pronounced standardization and appropriate QCs to attain a high level of reproducibility and sufficient reflection of the MIC of the examined antimicrobials. The main differences between these are the potency of discs (content of antimicrobial) and agar used, which results in different breakpoints for the susceptibility categorization. The CLSI disc diffusion method (*45*) and the British Society for Antimicrobial Chemotherapy disc diffusion method (BSAC; www.bsac.org.uk) are methods that have been used and recommended in several regions (*46*). This WHO manual describes the Calibrated Dichotomous Sensitivity (CDS) disc diffusion test method (*47, 48*). The CDS method is used for gonococcal antimicrobial susceptibility testing and surveillance, e.g. in the WHO Western Pacific and South-East Asia Regions. NOTE: The agar medium, procedure, and interpretative criteria of the CDS method differs from the other disc diffusion methods. If these methods are performed, their instructions need to be strictly followed.

4.8.6.2 *Recommended agar medium for the CDS method*

The test medium used in the CDS method for *N. gonorrhoeae* isolates is chocolate agar comprising Columbia agar base (Oxoid, is suitable; other commercial brands should be evaluated for their quality) with 8% horse blood "chocolated" at 70°C for 30 minutes (see

Table 4.11: Interpretative criteria for categorizing *N. gonorrhoeae* **into susceptibility categories using the CDS method; the annular radius in mm and not the zone diameter should be measured.**

Category of susceptibility				
Antibiotic	Disc content	Resistance	Decreased susceptibility	Susceptibility
Benzylpenicillin	0.5 IU	<3 mm	3–9 mm	>9 mm
Azithromycin	15 µg	<8 mm	—	≥8 mm
Ciprofloxacin testing[a]				
– Nalidixic acid	30 µg	0 mm	0 mm	>6 mm
– Ciprofloxacin	1 µg	≤6 mm	>6 mm	>6 mm
Ceftriaxone testing[b]				
– Ceftriaxone	0.5 µg	TBD[c]	5–9 mm	>9 mm
– Cefpodoxime	10 µg	TBD[c]	≤12 mm	>12 mm
Spectinomycin	100 µg	<6 mm	—	≥6 mm

[a] For testing fluoroquinolone (ciprofloxacin) susceptibility, both the nalidixic acid and ciprofloxacin disc should be used and simultaneously interpreted.

[b] For testing ceftriaxone susceptibility, both the ceftriaxone and cefpodoxime disc should be used and simultaneously interpreted.

[c] TBD, to be determined. Due to the lack of adequate number of resistant strains and evidence-based correlates between the MIC of isolates and treatment outcome, the breakpoints can not be determined yet. Isolates with decreased susceptibility/resistance to ceftriaxone should be confirmed by MIC testing (agar dilution or Etest) by local laboratory or ideally at a WHO Reference Laboratory.

Annex 4). Other media have not been validated and should not be used without a strict evaluation and probable adjustment of the interpretative criteria.

4.8.6.3 Interpretative criteria for the CDS method

Table 4.11 describes interpretative criteria for susceptibility, decreased susceptibility, and resistance that are recommended using the CDS method (*48*).

4.8.6.4 Quality control (QC) of the CDS method

N. gonorrhoeae reference strains WHO C, WHO K, and WHO P (if azithromycin is examined) should be included with each batch of antimicrobial susceptibility testing and each time a new batch of agar medium or discs are used. The annular radius to each antimicrobial and reference strain should be documented on a QC chart. For accepted annular radii in the QC, see Table 4.12.

4.8.6.5 Procedure of the CDS method

1. Dry (free of visible moisture, but do not overdry the plates) the number of chocolate agar plates (see Annex 4) needed. Dry plates inverted, with the lids removed, at 36±1°C, for 1 hour.

2. Sample most of a *N. gonorrhoeae* colony (at least 1–2 mm diameter grown overnight) on non-selective gonococcal agar medium with a plastic loop (1 µl) or straight nichrome wire (after flaming and cooling down). If only small colonies (<1 mm) are available, it may be necessary to collect 3–5 colonies before material is visible on the tip of the straight wire.

3. Prepare suspension in 2.5 ml sterile saline solution (approximately 10^7 CFU per ml) by rotating the straight wire at least 10 times with the tip in contact with the bottom of the test tube. Confirm that the material has come off the tip, mix the inoculum at least 10 times using a sterile Pasteur pipette and check that it is a homogenous solution. A too heavy inoculum will cause a slight decrease in zone sizes. A light inoculum will cause a marked increase in zone sizes.

4. Use the same pipette to transfer all the suspension to the surface of the pre-dried chocolate agar plate.

5. Distribute inoculum by rocking and check that the suspension covers the whole agar surface.

6. Remove excess inoculum with sterile Pasteur pipette.

7. Allow plate to dry for approximately 10–15 minutes at room temperature (plates should not be left longer than 15 minutes after the inoculum has dried).

8. Apply antimicrobial discs (brought to room temperature) on the inoculated plate using sterile forceps or with a disc dispenser. Up to six discs can be applied on a single 90 mm plate. Discs should be applied evenly and should not be removed after initial contact with the agar.

9. Incubate the plates for 18 hours at $36\pm1°C$ in $5\pm1\%$ CO_2-enriched atmosphere (70–80% humidity). CO_2 incubator or candle extinction jar with additional humidity can be used.

10. Measure annular radii with plastic mm ruler or vernier callipers (Fig. 4.12). The results of the WHO reference strains, used as QC, must be read and approved before reading other results (see Table 4.12). If not approved, perform troubleshooting and repeat the testing.

11. Interpret zone sizes into category of susceptibility (see Table 4.11).

4.8.7 Detection of plasmid-mediated resistance to penicillin

N. gonorrhoeae may carry plasmids that produce an enzyme (β-lactamase [penicillinase]) that inactivates penicillins such as benzylpenicillin, penicillin, ampicillin, and amoxicillin. Several qualitative methods have been used to detect β-lactamase production by microorganisms.

The chromogenic cephalosporin method is simple, sensitive, specific, and widely used to detect β-lactamase in gonococci. When the β-lactam ring of the chromogenic cephalosporin, nitrocefin, is hydrolysed by β-lactamase, a colour change from yellow to red occurs. The test is available commercially in a variety of formats, and lyophilized nitrocefin also can be purchased for in-house tests (*49*). Other less standardized and quality-assured methods to detect β-lactamase, such as the Acidometric method and the Iodometric test, also exist.

4.8.7.1 Nitrocefin disc method

1. Hydrate a nitrocefin disc on a glass slide or in a clean empty plate with sterile distilled water.

2. Sample several colonies from overnight pure gonococcal culture with a sterile loop and streak onto the surface of the disc.

3. A positive reaction usually produces red colour within 1 minute. However, weak positive reactions may take slightly longer to develop, but this is very rare. A negative result will show no colour change (remains yellow).

4.8.7.2 Nitrocefin solution method

The nitrocefin solution method is performed either by dropping the reagent directly onto colonies growing on selective or non-selective media or by inoculating the solution on a glass slide/filter paper with colonies.
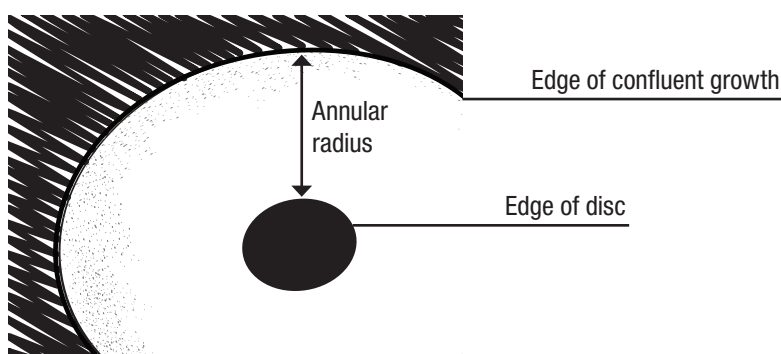

Figure 4.12
The annular radius (mm) is the shortest measured distance from the edge of the disc to the edge of confluent growth. The edge of confluent growth usually corresponds to the sharpest edge of the zone of inhibition.
Source: Reproduced from the CDS Antibiotic Susceptibility Test (http://web.med.unsw.edu.au/cdstest/).

Table 4.12: Antimicrobials, disc potencies, and acceptable annular radii of inhibition for WHO *N. gonorrhoeae* **reference strains recommended for quality control (QC)**

Strain/antimicrobial	Disc potency	Annular radii (mm)
N. gonorrhoeae ACM 5239 (WHO C)		
Azithromycin	15 µg	8.9–12.5
Benzylpenicillin	0.5 IU	2.1–4.1
Cefpodoxime	10 µg	10.9–14.5
Ceftriaxone	0.5 µg	8.2–11.0
Ciprofloxacin	1 µg	12.7–16.3
Nalidixic acid	30 µg	11.3–14.5
Spectinomycin	100 µg	6.9–8.9
N. gonorrhoeae WHO K		
Cefpodoxime	10 µg	7.6–11.1
Ceftriaxone	0.5 µg	7.3–9.3
N. gonorrhoeae WHO P		
Azithromycin	15 µg	3.8–7.4

Commercially available nitrocefin solution or a solution prepared in-house using nitrocefin powder can be used.

Direct plate test:

1. Add one drop of nitrocefin solution directly onto pure isolated gonococcal colonies on the agar medium. The agar dilution or Etest agar plates may be used after reading the MICs of examined antimicrobials.

2. A colour change of the nitrocefin solution from yellow to red within 1 minute indicates a positive result. However, very rare weak positive reactions may take slightly longer to develop. A negative result will show no colour change (remains yellow) (Fig. 4.13).

Slide/filter paper test:

1. Add one drop of nitrocefin solution onto a clean glass slide/filter paper.

2. Sample several colonies from overnight pure gonococcal culture with a sterile loop and emulsify into the nitrocefin drop.

3. A colour change from yellow to red within 1 minute indicates a positive result. Very rare weak positive reactions may take slightly longer to develop. A negative result will show no colour change (remains yellow).

4.8.7.3 Acidometric method

Place a strip of filter paper in an empty, clean Petri dish. Saturate the paper with penicillin solution (0.05 mol/l phosphate buffer, pH 8.0, 0.2 g/l bromcresol purple, and 50 g/l buffer-free benzylpenicillin [store frozen]). With a bacteriological loop, spread 10–20 colonies over an area of approximately 5 mm of the filter paper. Incubate the inoculated filter paper at room temperature for 30 minutes with the Petri dish lid on. β-lactamase activity will result in a colour change from blue to yellow, usually visible in less than 10 minutes.

4.8.7.4 Iodometric test

A penicillin-iodine mixture is freshly prepared by adding 1.1 ml of an iodine solution (1.5 mg of potassium iodide and 0.3 g of iodine in 100 ml of 0.1 mol/l phosphate buffer, pH 6.4 stored in a brown bottle at 4°C) to a vial
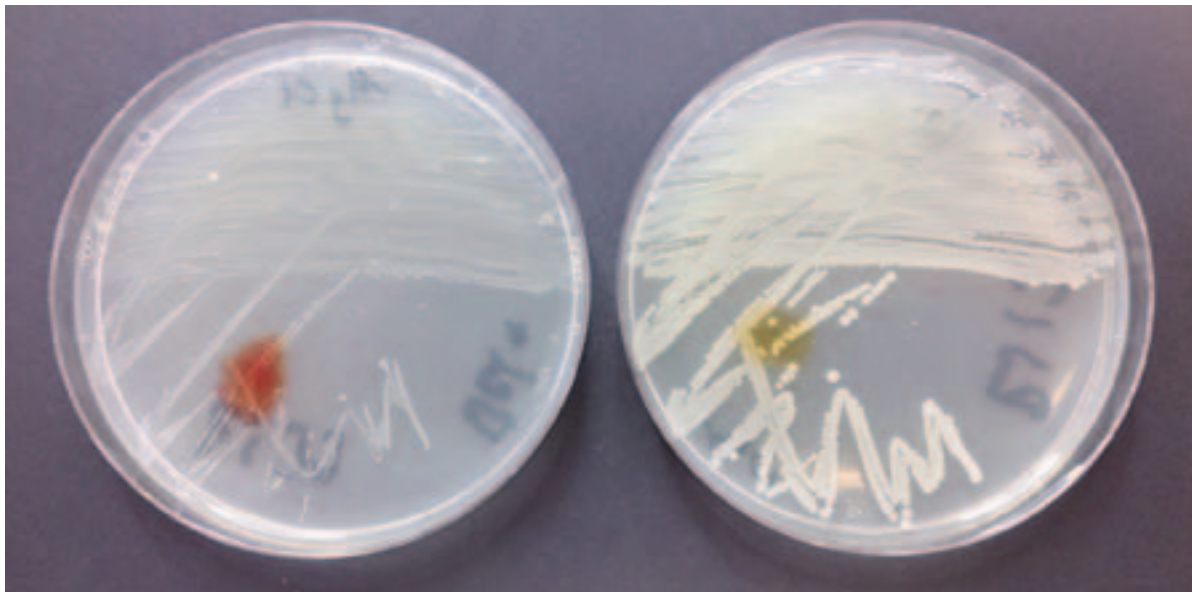
Figure 4.13
β–lactamase activity in *N. gonorrhoeae* determined on culture plate with chromogenic cephalosporin, nitrocefin. Left medium demonstrates positive reaction (red) and right medium shows negative reactions (remains yellow).

containing 0.15 ml of benzylpenicillin solution (1 million International Units per ml, stored at –20°C). The reagent mixture should be used within 1 hour. A loopful of the test organism is removed from colonial growth on an agar plate and emulsified in 1 drop of the penicillin-iodine mixture on a glass plate. One drop of a starch solution (4 g/l in distilled water, autoclaved and stored at 4°C) is added, giving a deep purple colour to the mixture. A negative result is indicated when this colour remains for 5 minutes. A colour change to colourless within 5 minutes (normally within 1 minute) indicates a positive test.

4.9 Preservation of *N. gonorrhoeae* isolates

To maintain the viability of *N. gonorrhoeae* strains on gonococcal agar media, it is necessary to subculture at least every 48 hours. Accordingly, effective methods for long-term preservation of gonococcal strains are crucial.

4.9.1 Conservation on chocolate agar slopes (*50*, *51*)

Storage up to 9 months (strains keep viable during transportation up to 5 days):

- A pure overnight culture on gonococcal agar media is heavily inoculated onto a 3 ml volume chocolate agar slope in a polycarbonate screw-capped Bijou bottle (5 ml volume; plastic bottle

must be used) and incubated with the screw-cap loosened for a minimum of 24 hours at 36±1°C, 5±1% CO_2-enriched atmosphere or until visible growth is present on the agar surface. Sterile liquid paraffin is then used to completely fill the agar slope, the screw-cap lid is fully tightened, and the Bijou bottle slope is then stored at 37°C. When the gonococci are required for testing, a sterile bacteriological loop is inserted through the paraffin overlay to remove some *N. gonorrhoeae* growth that is inoculated on gonococcal selective culture medium. After incubation, for 48 hours at 36±1°C, 5±1% CO_2-enriched atmosphere, gonococcal colonies are readily discernable and can be subcultured for appropriate examination (globules of paraffin also will be present but can easily be distinguished from the gonococcal colonies). The original paraffin overlaid slope can be returned to storage for further use.

4.9.2 Conservation by freezing or lyophilisation

Storage up to 1–3 months:

- All growth on a plate of pure overnight culture on gonococcal agar media can be inoculated in a small vial containing 0.5 ml of a sterile nutritive broth (e.g. nutrient broth, trypticase soy broth, brain-heart infusion broth) with 15–20% glycerol, suspended with

sterile pipette and immediately frozen at –20–25°C. Extended storage at this temperature is not desirable as gonococci will lose their viability.

Long-term storage:

- All growth on a plate of pure overnight culture on gonococcal agar media can be inoculated in a small vial containing approximately 0.5–1.0 ml of a sterile cryoprotective nutritive broth with 15–20% glycerol, suspended with sterile pipette and immediately frozen at –70°C.

- All growth on a plate of pure overnight culture on gonococcal agar media can be inoculated in a small cryovial containing sterile cryoprotective medium (e.g. nutritive broth with 15–20% glycerol), suspended with sterile pipette and immediately frozen in liquid nitrogen.

- All growth on a plate of pure overnight culture on gonococcal agar media can be inoculated in a small vial containing approximately 0.5–1.0 ml of a sterile microbank fluid with cryobeads (commercially available), suspended by inverting five times and, subsequently, as much liquid as possible is removed and the vial immediately frozen at –70°C.

- All growth on a plate of pure overnight culture on gonococcal agar media can be inoculated into sterile nutritive broth (e.g. nutrient broth, trypticase soy broth, brain-heart infusion broth) with 15–20% glycerol (alternatively, if no nutritive broth is not available, sterile skim milk can be used) and preserved by lyophilisation.

4.10 Retrieval of frozen *N. gonorrhoeae* isolates

Remove the cryotube containing the isolate from freezer or liquid nitrogen and do not allow it to thaw. Using the tip of a sterile Pasteur pipette, gently remove a small sample of the frozen bacterial suspension (or one cryobead) and transfer it to a gonococcal agar culture medium. Use a loop to streak the inoculum for single isolated colonies and incubate culture plate for 24 hours at 36±1°C, 5±1% CO_2-enriched atmosphere. Return the cryotube immediately to the freezer.

4.11 References

1. *Global incidence and prevalence of selected curable sexually transmitted infections*—2008. Geneva, World Health Organization, 2012 (http://www.who.int/reproductivehealth/publications/rtis/2008_STI_estimates.pdf, accessed 2 April 2013).

2. Tapsall JW et al. Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. *Expert Review of Anti-Infective Therapy*, 2009, 7(7):821–834.

3. Ohnishi M et al. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrobial Agents and Chemotherapy*, 2011, 55(7):3538–3545.

4. Bachmann LH et al. Nucleic acid amplification tests for diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* rectal infections. *Journal of Clinical Microbiology*, 2010, 48(5):1827–1832.

5. Mimiaga MJ et al. Gonococcal, chlamydia, and syphilis infection positivity among MSM attending a large primary care clinic, Boston, 2003 to 2004. *Sexually Transmitted Diseases*, 2009, 36(8):507–511.

6. Schachter J et al. Nucleic acid amplification tests in the diagnosis of chlamydial and gonococcal infections of the oropharynx and rectum in men who have sex with men. *Sexually Transmitted Diseases*, 2008, 35(7):637–642.

7. Masek BJ et al. Performance of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by use of self-collected vaginal swabs obtained via an Internet-based screening program. *Journal of Clinical Microbiology*, 2009, 47(6):1663–1667.

8. Hobbs MM et al. From the NIH: proceedings of a workshop on the importance of self-obtained vaginal specimens for detection of sexually transmitted infections. *Sexually Transmitted Diseases*, 2008, 35(1):8–13.

9. Taylor SN, DiCarlo RP, Martin DH. Comparison of methylene blue/gentian violet stain to Gram's stain for the rapid diagnosis of gonococcal urethritis in men. *Sexually Transmitted Diseases*, 2011, 38(11):995–996.

10. Martin JE, Lester A. Transgrow, a medium for transport and growth of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *HSMHA Health Reports*, 1971, 86(1):30–33.

11. Taylor E, Phillips I. Assessment of transport and isolation methods for gonococci. *British Journal of Venereal Disease*, 1980, 56(6):390–393.

12. Jephcott AE, Bhattacharyya MN, Jackson DH. Improved transport and culture system for the rapid diagnosis of gonorrhoea. *British Journal of Venereal Disease*, 1976, 52(4):250–252.

13. Thayer JD, Martin JE. An improved selective medium for cultivation of *N. gonorrhoeae* and *N. meningitidis*. *Public Health Reports*, 1966, 81:559–562.

14. Faur YC et al. A new medium for the isolation of pathogenic Neisseria (NYC medium). 1. Formulation and comparisons with standard media. *Health Laboratory Science*, 1973, 10:44–52.

15. Mirrett S, Reller LB, Knapp JS. *Neisseria gonorrhoeae* strains inhibited by vancomycin in selective media and correlation with auxotype. *Journal of Clinical Microbiology*, 1981, 14(1):94–99.

16. Unemo M et al. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. *Journal of Antimicrobial Chemotherapy*, 2009, 63(6):1142–1151.

17. Alexander S, Ison C. Evaluation of commercial kits for the identification of *Neisseria gonorrhoeae*. *Journal of Medical Microbiology*, 2005, 54(9):827–831.

18. Tapsall JW, Cheng JK. Rapid identification of pathogenic species of Neisseria by carbohydrate degradation tests. Importance of glucose in media used for preparation of inocula. *British Journal of Venereal Disease*, 1981, 57(4):249–252.

19. Dillon JR, Carballo M, Pauzé M. Evaluation of eight methods for identification of pathogenic Neisseria species: Neisseria-Kwik, RIM-N, Gonobio-Test, Minitek, Gonochek II, GonoGen, Phadebact Monoclonal GC OMNI Test, and Syva MicroTrak Test. *Journal of Clinical Microbiology*, 1988, 26(3):493–497.

20. Unemo M et al. Global transmission of prolyliminopeptidase-negative *Neisseria gonorrhoeae* strains: implications for changes in diagnostic strategies. *Sexually Transmitted Infections*, 2007, 83(1):47–51.

21. Ilina EN et al. Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic Neisseria. *Journal of Molecular Diagnostics*, 2009, 11(1):75–86.

22. Cook RL et al. Systematic review: noninvasive testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *Annals of Internal Medicine*, 2005, 142(11):914–925.

23. Alexander S. The challenges of detecting gonorrhoea and chlamydia in rectal and pharyngeal sites: could we, should we, be doing more? *Sexually Transmitted Infections*, 2009, 85(3):159–160.

24. Whiley DM et al. Exploring 'best practice' for nucleic acid detection of *Neisseria gonorrhoeae*. *Sexual Health*, 2008, 5(1):17–23.

25. Palmer HM et al. Evaluation of the specificities of five DNA amplification methods for the detection of *Neisseria gonorrhoeae*. *Journal of Clinical Microbiology*, 2003, 41(2):835–837.

26. Tabrizi SN et al. Evaluation of six commercial nucleic acid amplification tests for detection of *Neisseria gonorrhoeae* and other Neisseria species. *Journal of Clinical Microbiology*, 2011, 49(10):3610–3615.

27. McNally LP et al. Low positive predictive value of a nucleic acid amplification test for nongenital *Neisseria gonorrhoeae* infection in homosexual men. *Clinical Infectious Diseases*, 2008, 47(2):e25–27.

28. Golparian D, Tabrizi SN, Unemo M. Analytical specificity and sensitivity of the APTIMA Combo 2 and APTIMA GC assays for detection of commensal Neisseria species and *Neisseria gonorrhoeae* on the Gen-Probe Panther instrument. *Sexually Transmitted Diseases*, 2013, 40(2):175–178.

29. Whiley DM et al. A real-time PCR assay for the detection of *Neisseria gonorrhoeae* in genital and extragenital specimens. *Diagnostic Microbiology and Infectious Diseases*, 2005, 52(1):1–5.

30. Hjelmevoll SO et al. A fast real-time polymerase chain reaction method for sensitive and specific detection of the *Neisseria gonorrhoeae porA* pseudogene. *Journal of Molecular Diagnostics*, 2006, 8(5):574–581.

31. Tabrizi SN et al. Evaluation of *opa*-based real-time PCR for detection of *Neisseria gonorrhoeae*. *Sexually Transmitted Diseases*, 2005, 32(3):199–202.

32. Goire N et al. A duplex *Neisseria gonorrhoeae* real-time polymerase chain reaction assay targeting the gonococcal *porA* pseudogene and multicopy *opa* genes. *Diagnostic Microbiology and Infectious Diseases*, 2008, 61(1):6–12.

33. Whiley DM et al. False-negative results using *Neisseria gonorrhoeae porA* pseudogene PCR—a clinical gonococcal isolate with an *N. meningitidis porA* sequence, Australia, March 2011. *Eurosurveillance*, 2011, 16(21):pii:19874.

34. Eastick K, Winter A, Jamdar S. Identification of *Neisseria gonorrhoeae* isolates with a recombinant *porA* gene in Scotland, United Kingdom, 2010 to 2011. *Eurosurveillance*, 2012, 17(9):pii:20101.

35. Golparian D, Johansson E, Unemo M. Clinical *Neisseria gonorrhoeae* isolate with a *N. meningitidis porA* gene and no prolyliminopeptidase activity, Sweden, 2011—danger of false-negative genetic and culture diagnostic results. *Eurosurveillance*, 2012, 17(9):pii:20102.

36. Ison CA et al. Evolution of *Neisseria gonorrhoeae* is a continuing challenge for molecular detection of gonorrhoea: false-negative gonococcal *porA* mutants are spreading internationally. *Sexually Transmitted Infections*, 2013, 89(3):197-201.

37. Huppert J, Hesse E, Gaydos CA. What's the point? How point-of-care STI tests can impact infected patients. *Point of Care*, 2010, 9(1):36–46.

38. Gift TL et al. The rapid test paradox: when fewer cases detected lead to more cases treated: a decision analysis of tests for *Chlamydia trachomatis*. *Sexually Transmitted Diseases*, 1999, 26(4):232–240.

39. Yokoi S et al. Threat to cefixime treatment of gonorrhea. *Emerging Infectious Diseases*, 2007, 13(8):1275–1277.

40. Unemo M et al. Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. *Eurosurveillance*, 2010, 15(47):pii:19721.

41. Ison CA et al. Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. *Eurosurveillance*, 2011, 16(14):pii:19833.

42. Unemo M et al. First *Neisseria gonorrhoeae* strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011. *Eurosurveillance*, 2011, 16(43):pii:19998.

43. Unemo M et al. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. *Antimicrobial Agents and Chemotherapy*, 2012, 56(3):1273–1280.

44. Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future Microbiolog*y, 2012, 7(12):1401–1422.

45. Cockerill F et al. Performance standards for antimicrobial susceptibility testing: twenty-first informational supplement, M100-S21 and Vol. 31, No. 1. Wayne, PA, *Clinical and Laboratory Standards Institute*, 2011 (www.clsi.org).

46. Dillon J-A R, Starnino S. *Manual de laboratorio: identificación y evaluación de la susceptibilitad de Nesissería gonorrhoeae a los antibióticos para el programa de vigilancia de la susceptibilidad de los gonococos a los agentes antimicrobianos en America Latina y el Caribe* (GASP-LAC) (segunda edición). Canada, University of Saskatchewan, 2011 (http://www.gasp-lac.net).

47. Bell SM. The CDS disc method of antibiotic sensitivity testing (calibrated dichotomous sensitivity test). *Pathology*, 1975, 7(4 Suppl):Suppl 1–48.

48. The CDS antibiotic susceptibility test (http://web.med.unsw.edu.au/cdstest).

49. O'Callaghan CH et al. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. *Antimicrobial Agents and Chemotherapy*, 1972, 1(4):283–288.

50. Cody RM. Preservation and storage of pathogenic Neisseria. *Health Laboratory Science*, 1978, 15(4):206–209.

51. Tapsall JW, Phillips EA, Morris LM. Chromosomally mediated intrinsic resistance to penicillin in penicillinase producing strains of *Neisseria gonorrhoeae* isolated in Sydney: guide to treatment with Augmentin. *Genitourinary Medicine*, 1987, 63(5):305–308.

Chapter 5

Chlamydial infections

5.1 Introduction

Chlamydia trachomatis, the etiological agent of chlamydia, causes substantial morbidity and economic cost worldwide. In 2008, the World Health Organization (WHO) estimated 106 million new cases of urogenital chlamydia among adults globally. This places chlamydia as the most prevalent bacterial sexually transmitted infection (STI) together with gonorrhoea (also 106 million new cases).

C. trachomatis has been classified into three biovars, each containing several serovars or genotypes (dependent on method used for classification). The biovars were defined based on the type of infection, common localization of infection (tissue tropism), and relative virulence of the disease (Table 5.1)—those that cause trachoma (the leading cause of preventable blindness worldwide and endemic in many developing countries), those that cause genital infection (a leading bacterial STI globally), and those that cause lymphogranuloma venereum (LGV; a genital ulcer disease that affects lymphoid tissue) (see Chapter 11).

The ocular biovar consists of serovars A–C, which are found predominantly in conjunctival infections. The tissue tropism is not absolute as these organisms, especially serovar B, also may be isolated from genital infections, but the frequency of these occurrences is rare.

The predominant biovar consists of serovars D–K, which are transmitted sexually and infect the genital epithelium, causing urethritis in men and cervicitis (and urethritis) in women (Table 5.2). Asymptomatic urogenital infection occurs in up to 50% of men and up to 90% of women. If undetected and not treated, infection can ascend to the upper genital tract and cause epididymitis in men and pelvic inflammatory disease (PID) and related sequelae (ectopic pregnancy and tubal factor infertility) in women. These serovars may also be isolated from ocular infections in neonates, acquired during passage through an infected birth canal, but are not largely responsible for trachoma. Neonates also may develop chlamydial

pneumonia because of exposure to these serovars during vaginal delivery (not to be confused with infection with *C. pneumoniae*).

Finally, the LGV biovar, consisting of serovars L1–L3, is also a sexually transmitted biovar but with tissue preference for lymphoid cells and more aggressive disease progression. LGV infections are accordingly more invasive and likely to cause systemic infection than the other biovars. LGV is endemic in many developing world settings worldwide. Since 2003, outbreaks of LGV proctitis and proctocolitis have been documented among men who have sex with men (MSM) in Europe and North America, which previously only observed sporadic cases (see Chapter 11).

- *C. trachomatis* causes the most common bacterial STI globally, which includes a spectrum of diseases in a variety of sites (i.e. genital, ocular, lymph nodes, and bronchial).
- Negative outcomes associated with untreated *C. trachomatis* infections include PID, ectopic pregnancy, tubal factor infertility, epididymitis, prostatitis, and others.

5.2 Overview of available diagnostic methods

Although chlamydial infections are highly prevalent, the knowledge regarding this STI was highly limited in public health settings prior to the 1980s due to limitations with diagnostic methods. Early diagnostic techniques were developed around trachoma control efforts rather than the STIs. Serology was used to distinguish between acute and chronic infection, but lacks appropriate sensitivity and specificity for diagnosis of acute infection, and to obtain population-based estimates of lifetime exposure. Culture was standardized in the 1970s, making isolation of the organism a useful diagnostic tool.

Table 5.1: Characteristics and infections associated with different serovars of _C. trachomatis_

Serovar	Characteristics	Tissue tropism/Biovar	Infection
A–C (Incl. Ba)	Non-invasive	Epithelial cells/Trachoma	Endemic blinding trachoma
D–K (Incl. Da, Ia, Ja)	Non-invasive	Epithelial cells/Trachoma	Urogenital, conjunctivitis, neonatal pneumonia
L1, L2, L3 (Incl. L2a, L2b)	Invasive	Lymphatic cells/LGV	LGV

LGV, lymphogranuloma venereum.

Table 5.2: Clinical manifestations of infection with _C. trachomatis_

Genital infection	Primary	Sequelae
Women	Cervicitis, copious purulent discharge, firable cervix, dysuria, pelvic pain, cervical motion tenderness	Pelvic inflammatory disease, ectopic pregnancy, salpingitis, tubal factor infertility
Men	Urethral discharge, dysuria, testicular pain	Epididymitis, prostatitis
Non-genital infections	**Primary**	**Sequelae**
Rectal	Discharge, rectal pain, blood in stool	Proctitis
Oropharyngeal	Pharyngitis, mild sore throat	
Lymph nodes	Lymphatic inflammation	
Ocular	Conjunctivitis	Scarring, blinding trachoma
Neonatal pneumonia	Pneumonia	

Note: Studies have provided evidence that _C. trachomatis_ infection may facilitate HIV transmission; however, the odds ratios have been relatively low.

However, the need to maintain organism viability requiring stringent transportation and storage conditions limited the availability of this test to clinics working with more developed laboratories. Antigen detection assays, direct immunofluorescence assays (DFAs), and solid phase enzyme-linked immunosorbent assays (ELISAs) were developed in the early 1980s, making diagnosis of chlamydial infections more available. As additional ELISAs were developed, several rapid, point-of-care (POC) assays became available. DFA, lab-based ELISA, and POC suffered from low sensitivity relative to culture and suboptimal specificity. However, the rapid turnaround time and the less restrictive requirements for specimen transportation made them an attractive option, particularly in high-prevalence settings.

The next major advance in chlamydia diagnostics was the utilization of nucleic acid sequences rather than antigens as detection targets. The sensitivity of the initially developed non-amplified nucleic acid hybridization assay (NAH) tests was similar to that of culture, but again there were questions regarding specificity. The addition of _Neisseria gonorrhoeae_ to the test menu made this assay extremely attractive. Nucleic acid amplification tests (NAATs) were the next diagnostic development. NAATs use enzymatic methods to amplify target DNA or RNA exponentially into billions of copies. Amplified product is detected by a variety of means, giving each assay unique performance characteristics. Similar to the NAH test, NAATs combined chlamydia and gonorrhoea testing from a single sample. Due to the

superior performance characteristics, e.g. sensitivity, specificity, range of specimen types, automation, and independence from maintaining organism viability, NAATs are strongly recommended for diagnosis and screening of chlamydial infections. For additional information regarding performance characteristics of different methods for diagnosis of chlamydia, see the chlamydiae web site, www.chlamydiae.com.

Issues associated with assay evaluation and performance variability (1, 2) underscore the need for verification of assays and strict quality assurance (QA) within each laboratory, not only prior to adoption of a method, but also on a recurring basis. For further information on this topic, see Chapter 2 and Annex 3. For adequate performance characteristics of all diagnostic methods, it is crucial to follow precisely the recommendations from the manufacturer concerning collection, transportation, and storage of samples, as well as performance of the specific assay, including quality controls.

- Chlamydia diagnostic technologies continue to advance and provide improved sensitivity.

- Due to the superior performance characteristics, NAATs are strongly recommended for diagnosis and screening of chlamydial infections. However, the choice of tests is dependent on the resources available and level of laboratory support.

5.3 Collection, transportation, and storage conditions of specimens

The collection, transportation, and storage conditions of specimens (Table 5.3) have become assay-dependent in many cases. This section will present some general guidelines, but details related to any specific diagnostic assay should be taken from the appropriate package insert instructions.

The anatomic sampling sites will vary depending on the clinical presentation and history of the patient and the overall sensitivity of the assay. For culture, which requires live organisms, the sample must be collected from sites with columnar or cuboidal epithelial cells, which are most likely to be actively infected. Thus, the endocervical os should be sampled in women and the

urethral epithelium should be sampled in men and, if appropriate, in women. Studies have shown repeatedly infections isolated to the endocervix or to the female urethra. Culturing both of these sites when testing women will increase case finding.

Obtain endocervical samples by inserting the collection device 2–3 cm into the os and rotating a full 360°. Endocervical samples are not taken in girls of prepubertal age; instead, specimens should be sampled from the vestibule of the vagina, and a urine specimen should also be sampled. Collect urethral samples by inserting the swab 2–3 cm into the urethra, followed by full rotation to obtain cellular material. Sample inadequacy has been described as a common problem that will negatively affect culture sensitivity.

While other non-culture, non-NAAT assays do not require viable organisms, the limited sensitivity of these assays requires that sufficient organisms be collected to obtain a positive result. Additionally, antigen detection methods generally require predominantly intact organisms. Thus, the same sampling restrictions as necessary for culture generally apply to these diagnostic methods. Some ELISAs have been approved for use with male urine samples since this sampling method washes the organisms from the urethra. If the volume of first-catch urine is carefully controlled (less than 25 ml in general), and the patient has not voided during the previous hour, the organism load is likely to be sufficiently concentrated to be detectable in these assays. This is not true for female urine since the occurrence of urethral infections is less common and the urine does not sample the cervical tissue.

NAATs have the advantage of reliance on nucleic acid materials that do not require viable or intact organisms. As a result, these assays have expanded our ability to collect less invasive samples that require clinical settings. NAATs are highly sensitive when using self-obtained samples (e.g. vaginal swabs or male urine). NAATs also are useful for testing residual liquid-based cytology medium, allowing screening to be performed for women receiving Papanicolaou (Pap) testing. Vaginal swabs may be patient- or clinician-obtained with equal utility (3, 4). Insert swabs into the vagina and rotate to sample the vaginal walls completely.

Non-genital sampling is required in some cases, i.e. dependent on clinical signs and sexual practice. Culture can identify rectal and oropharyngeal infections, but the sensitivity is quite low due to contaminating microflora. However, NAATs have higher sensitivity for these infections despite no manufacturer's claim for these sample types. Use of NAATs with extragenital samples in MSM increases case finding substantially (5–8). Sample rectal specimens, clinician- or patient-obtained, by inserting a Dacron swab as far as comfortable (2–3 cm) into the rectum and turning 360°. There is no longer a need to visualize the rectum to collect an adequate sample due to the increased sensitivity of NAAT assays. For anyone reporting receptive anal intercourse, rectal testing is warranted. Self-collection of samples for use with NAATs may increase uptake of screening and reduce the burden of clinical time (9, 10). Collect oropharyngeal samples from the posterior pharynx and the tonsillar crypt. This sample type should be collected when diagnosing infections transmitted during oral sex. For samples collected from neonates suspected of having contracted chlamydia pneumonia during delivery, obtain a nasopharyngeal sample. This sample will be used for culture or DFA and has not been rigorously evaluated in any other systems. However, due to the low bacterial load, NAATs are likely to be most effective; more research is needed in this area. Insert the swab through the nostril until the pharyngeal wall can be sampled. Collect *conjunctival sample* by retracting the inferior eyelid and using a swab moving across the surface of the inferior palpebral conjunctiva towards the median corner of the eye. For suspected LGV, see Chapter 11.

Table 5.3: Sample collection, transportation, and storage

Site	Collection device	Sampling procedure	NAAT	Culture	DFA	POC
Endocervix	Swab/plastic,[a] liquid cytology brush or broom	Use a cleaning swab to remove excess mucus prior to sample collection. Broom collection is valid for NAAT only. Insert collection device 2–3 cm and rotate swab 360° in the endocervical os. Collection of endocervical cells is critical to DFA procedure.	Place into manufacturer's collection device or use liquid cytology medium. Store and transport according to package insert directions.	Place directly into appropriate chlamydia transport medium (e.g. SPG buffer with antimicrobial inhibitors). Maintain at 4°C for inoculation within 24 h or –70°C for longer storage.	Roll onto slide (thin layer) and air dry.	Place into kit extraction buffer and follow package insert directions.
Urethra	Swab/aluminium[b]	Insert swab 2–3 cm into the urethra and rotate 360°. Collection of cuboidal epithelial cells is critical for DFA.	Place into manufacturer's collection device. Store and transport according to package insert directions.	Place directly into appropriate chlamydia transport medium (e.g. SPG buffer with antimicrobial inhibitors). Maintain at 4°C for inoculation within 24 h or –70°C for longer storage	Roll onto slide (thin layer) and air dry.	Place into kit extraction buffer and follow package insert directions.

Site	Collection device	Sampling procedure	NAAT	Culture	DFA	POC
Urine	Sterile urine cup	Do not have patient clean the genital area. Obtain first portion of the void (less than 25 ml in general).	Place into manufacturer's collection device. Store and transport according to package insert directions.	NA	NA	NA
Vagina	Swab/plastic[a]	Clinicians or patients can obtain samples. Rotate the swab to come into contact with the vaginal walls on all sides.	Place into manufacturer's collection device. Store and transport according to package insert directions. Many assays can support "dry swabs" received in no medium.	NA	NA	Follow package insert directions.[c]
Rectum	Swab/plastic[a]	Insert swab 2–3 cm into the rectum and rotate 360°.	No manufacturer currently has a claim for this sample type.[d] Treat as an endocervical sample. Samples may be sent with no media if the assay in use can support this sample type for endocervical specimens.	Place directly into appropriate chlamydia transport medium (e.g. SPG buffer with antimicrobial inhibitors). Maintain at 4°C for inoculation within 24 h or –70°C for longer storage.	Roll onto slide (thin layer) and air dry	NA

Table 5.3: Sample collection, transportation, and storage (*continued*)

Site	Collection device	Sampling procedure	NAAT	Culture	DFA	POC
Oropharynx	Swab/plastic[a]	Swab the posterior pharynx and the tonsillar crypt.	No manufacturer currently has a claim for this sample type.[d] Treat as an endocervical sample. Samples may be sent with no media if the assay in use can support this sample type for endocervical specimens.	Place directly into appropriate chlamydia transport medium (e.g. SPG buffer with antimicrobial inhibitors).	Roll onto slide (thin layer) and air dry	NA
Nasopharynx (for suspected cases of neonatal pneumonia)	Swab/aluminium[b]	Swab the nasopharynx or take tracheobronchial aspirate.	NA	Place directly into appropriate chlamydia transport medium (e.g. SPG buffer with antimicrobial inhibitors). Maintain at 4°C for inoculation within 24 h or –70°C for longer storage.	NA	NA
Conjunctiva	Swab/aluminium[b]	Swab the surface of the inferior palpebral conjunctiva.	NA	Place directly into appropriate chlamydia transport medium (e.g. SPG buffer with antimicrobial inhibitors). Maintain at 4°C for inoculation within 24 h or –70°C for longer storage.	Roll onto slide (thin layer) and air dry	NA

DFA, direct immunofluorescence assays; NA, not applicable; NAAT, nucleic acid amplification test; POC, point-of-care; SPG, sucrose-phosphate-glutamate.

[a] Dacron or rayon swabs on a plastic shaft.

[b] Dacron or rayon swabs on an aluminium shaft.

[c] Only some POC tests are licensed for vaginal specimens.

[d] Data indicate that appropriate NAATs perform well for these sample types, but no manufacturer has a claim for extra-genital specimens.

Given the rigorous evaluation required before approval of a diagnostic test by the United States of America Food and Drug Administration (FDA), which includes multi-site clinical trials with comparisons against appropriate standards, the performance of FDA-approved assays is well documented. Therefore, we use this level of evaluation as the standard for determination of high-quality assays. Table 5.4 summarizes the performance characteristics of different FDA-cleared diagnostic tests for detection of *C. trachomatis*.

- Specimen collection, transportation, and storage conditions vary by detection assay and may have a significant influence on the sensitivity of the testing.
- Selection of appropriate specimen and detection assay are crucial for effective diagnosis.

Table 5.4: Evaluated* diagnostic tests (as of June 2012) for detection of *C. trachomatis*

	NAAT	Culture	DFA	POC
Specimen types				
Endocervical swab	Yes	Yes	Yes	Yes
Liquid cytology medium	Yes (some tests)	No	No	No
Vaginal swabs				
Self-obtained	Yes (some tests)	No	No	Yes (some tests)
Clinician-collected	Yes (some tests)	No	No	Yes (some tests)
Urine				
Female	Yes	No	No	No
Male	Yes	No	No	No
Male urethral swab	Yes	Yes	Yes	Yes
Rectal swab	No[a]	Yes[b]	Yes[b]	No
Oropharyngeal swab	No[a]	Yes[b]	Yes[b]	No
Conjunctival swab	No[a]	Yes	Yes	No
Performance				
Sensitivity[c]	Very high	Moderate–high	Low–moderate	Low–moderate
Specificity	Very high	Very high	Moderate	Very high
Other considerations				
Cost	Very high	Moderate	Low	Low
Transportation and storage	Ambient up to 60 days (check package insert)	4°C for 24 h −70°C after 24 h	Ambient	NA
Instrumentation	Large footprint	Routine microbiology/ virology	Fluorescent microscope	Small–none
Throughput/ automation	High/yes	Low/no	Low/no	Low/no

Table 5.4: Evaluated* diagnostic tests (as of June 2012) for detection of *C. trachomatis* (*continued*)

	NAAT	Culture	DFA	POC
Technical complexity	High	High	Moderate (microscopy skills)	Low
Level of laboratory infrastructure	Reference	Reference	Central	Site
Multiple pathogens from one sample	*N. gonorrhoeae*, *Trichomonas vaginalis* and HPV on some platforms	No	No	No
Other comments	• Due to the superior performance characteristics, NAATs are strongly recommended for diagnosis and screening • Potential for laboratory contamination requires strict adherence to protocols • Some require large batch size, which may delay turnaround time	• Strict collection and transportation is crucial to maintain viability—this is the major barrier to sensitivity with this assay • Potential to obtain viable isolates is useful for additional testing such as genotyping and antibiotic susceptibility testing	• Highly recommended for immediate identification of conjunctival infections	• Infections identified may be treated before the patient leaves the clinic

DFA, direct immunofluorescence assay; HPV, human papillomavirus; NAAT, nucleic acid amplification test; POC, point-of-care.

* Evaluated by the United States of America Food and Drug Administration.

[a] Data indicate that appropriate NAATs perform well for these sample types, but no manufacturer has a claim for extra-genital specimens.

[b] Compared to modern NAATs, the sensitivity of culture and DFA assay is likely to be even lower for these specimen types.

[c] Sensitivity estimates vary widely depending on the different sensitivity of assays of the same methodology as well as assays used for comparison (the "gold standard"). The NAATs are superior to any other class of test for sensitivity and have excellent specificity.

Source: Adapted from Unemo M, Papp JR. Infections caused by *Chlamydia trachomatis*. In: Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010:40–63.

5.4 Nucleic acid amplification tests (NAATs)

Molecular detection of specific nucleic acid sequences and subsequent validation and commercialization of such assays have vastly improved laboratory detection of *C. trachomatis*. NAH tests such as Gen-Probe PACE 2 and PACE 2C (Gen-Probe, USA), which rely on the binding of specific complementary nucleic acid probes and subsequent signal amplification to detect binding, were the first developed. These tests are approximately 10–15% less sensitive than NAATs and should not be used if NAATs are available and affordable. NAATs are considered to have superior performance characteristics compared to any other test types for detection of chlamydial infections (*11–14*) and, as such, are the assay type recommended by the United States of America Centers for Disease Control and Prevention (CDC) (and

other regulatory and advising bodies) for both diagnosis, of genital and extragenital sites, and screening for chlamydial infections (*15*). Confirmatory testing of *C. trachomatis*-positive samples is no longer recommended. Validated and effective NAATs have several advantages that are independent of manufacturer. They are clearly the most sensitive assays available, which also creates opportunities of sample pooling in resource-limited settings; they are independent from maintaining organism viability; most commercially available assays can test for both chlamydia and gonorrhoea; and these assays have a broader range of useful sample types that include less invasively collected urine samples or vaginal swabs in addition to the previously required endocervical and urethral swabs. Additionally, these assays are well suited to automation, which results in increased standardization and QA of extraction and detection, as well as significantly increased throughput. However, NAAT assays also share some common difficulties. Amplified technologies require substantial instrumentation and are not appropriate to many laboratory settings in resource-constrained countries. These assays also are susceptible to environmental contamination due to the exponential amplification of target sequences. Any action that involves an open amplified sample is a potential source for aerosol formation and environmental contamination. Once laboratory facilities and equipment (e.g. pipettors) become contaminated, recovering from carry-over contamination may be extremely difficult.

Another issue involves the potential for false-negative results. The nucleic acid extraction process is critical to the process but is difficult to guarantee for each sample. Amplification requires precise salt, nucleotide, and enzyme concentrations to proceed efficiently, which requires high precision in pipetting. The enzyme that promotes amplification also may be sensitive to components of blood, mucus, and urine. Therefore, a negative result may actually reflect a lack of target nucleic acid due to inadequate sample collection or extraction or a lack of amplification rather than a lack of target sequence. The proportion of samples that are inhibitory can vary by test method and, using some methods, may be as high as 7.5% in certain populations (*16*). However, the performance of these tests is still strongly preferable to that of culture, which can provide

false-negative results due to loss of organism viability, and non-culture methods, which have sensitivity lower than (or maximum equal to) culture while also having suboptimal specificity. Therefore, despite the need for appropriate laboratory caution, NAATs advantages substantially outweigh the disadvantages. Use of NAATs should be encouraged even in resource-constrained settings by creation of regional reference laboratories that can provide diagnostic services using these methods. Regional reference laboratories provide many advantages that result from larger testing volumes, rigorous adherence to good laboratory practices, and improved technical expertise. Use of regional laboratories may reduce costs while using assays with the highest sensitivity. Despite the need to send samples out to a regional facility, the high volume and access to high-throughput automation may result in equivalent or reduced turnaround times. For these reasons, laboratories that cannot afford to perform the most sensitive NAAT assays should consider utilizing the services of a regional reference laboratory rather than using a less sensitive diagnostic tool.

Four companies currently have FDA-approved commercially available NAATs for the detection of *C. trachomatis*. The assays marketed by these manufacturers are described below. All commercially available and approved NAATs detect LGV as *C. trachomatis*-positive; however, without distinguishing the results as L1–L3 positive. For this purpose, genotyping is necessary (see Chapter 11). It is important to note that globally there are many additional commercially available or even laboratory-developed *C. trachomatis* NAATs in use (*17, 18*). If non-FDA approved NAATs are used, regional (such as European Union [EU]) and/or other national regulatory processes should provide safeguards on the quality and performance of the diagnostic NAAT. Use of internationally approved NAATs is strongly recommended. If this is not possible, it is strongly recommended that the effectiveness of the proposed NAAT for the local settings is strictly validated and quality-assured before use against at least one internationally approved NAAT and subsequently used with appropriate positive, negative, and inhibition controls; participation in appropriate external quality assessment system is strongly recommended as well

(see Chapter 2 and Annex 3). For basic information regarding different NAAT technologies, see Annex 3.

- Molecular technologies offer the best sensitivity with excellent specificity.
- Molecular assays reduce the need for strict transportation and storage conditions and remove subjective analysis (e.g. microscopy).
- If internationally approved NAATs cannot be used, it is strongly recommended that the effectiveness of the proposed NAAT for the local settings is strictly validated and quality-assured before use against at least one internationally approved NAAT.
- New assays are becoming available rapidly and cannot be anticipated in this document. It is important to assess the relevant literature continually for high-quality evaluations of new assays to determine the best fit for each laboratory.

5.4.1 Abbott molecular assays

The Abbott RealTime CT/NG and CT-only assays are run on the automated m2000 system (Abbott Molecular, USA). This assay has replaced the FDA-approved LCx assay, which was one of the first commercially available NAATs. The RealTime polymerase chain reaction (PCR) assay utilizes the m2000sp, an automated sample preparation instrument that extracts DNA using a magnetic particle-based capture system. Following extraction, the m2000sp instrument loads the master mix into the PCR tray, adds the purified samples, and they are ready for real-time amplification and detection in the m2000rt (*19*). This real-time thermal cycler detects amplification by fluorescence emitted when probes bind specifically to amplified target sequences during each amplification cycle. The assay now includes dual targets, i.e. two sequences on the cryptic plasmid (7–10 copies per organism), for *C. trachomatis* to ensure detection of the Swedish new variant of *C. trachomatis* (nvCT), which caused thousands of false-negative reports in Sweden and some other Nordic countries using NAATs at that time available from Roche and Abbott (*20–22*). The system can detect multiple signals that allow the assay to detect chlamydia, gonorrhoea, and a non-competitive internal control to measure

potential inhibition in each sample. The internal control sequence is based on plant DNA that is added during the DNA extraction step to provide a measure of extraction efficiency and potential PCR inhibition. This is the only assay that provides an appropriate indication that DNA isolation has been successful. The system is capable of processing 96 samples per run, including 3 controls, allowing 186 samples and 6 controls to be tested in approximately 8 hours.

Approved samples include urine, vaginal, endocervical, and urethral swabs. Swab samples and urine samples are collected using the multi-Collect kit provided by the manufacturer and are stable at 2–30°C for up to 14 days prior to testing. The kit is a single collection device for all sample types that can all run simultaneously on the m2000 system. The long stability of swabs and urines at ambient temperature makes this assay attractive for public health settings that ship samples to a reference laboratory.

The RealTime CT/NG assay has excellent analytic sensitivity and compares well even with the APTIMA Combo 2 assay (*23*). The DNA purification process is designed to remove potential inhibitors while the internal control provides a warning if inhibition exists. The process also is designed to remove naturally occurring fluorophores that might interfere with test performance. The use of homogeneous fluorescence detection technology with specific PCR primers combines the amplification and detection in a one-step closed system. The closed nature of the system reduces the potential for carryover contamination and use of the negative control provides the user with a method for rapidly identifying environmental contamination.

5.4.2 Becton, Dickinson (BD) diagnostic assays

The FDA-approved BD ProbeTec ET chlamydia and gonorrhoea assay (Becton, Dickinson and Company, USA) was the first commercially available real-time assay. This test uses isothermal strand displacement amplification (SDA) to amplify and detect target sequences simultaneously at 52.5°C. Amplification of a sequence of the chlamydial cryptic plasmid occurs in a sealed plate with a fluorescent energy transfer read-out. This scheme was designed to minimize the potential for environmental carry-over contamination since amplified samples are never opened.

Approved samples for the ProbeTec tests include urine, endocervical, and urethral swabs. Again, evidence suggests that vaginal swabs are a useful sample type and studies are underway investigating the performance of the assay with additional sample types. Swab samples are collected using the kit provided by the manufacturer and are stable at room temperature for up to 6 days prior to testing. First-catch urine is stable for up to 24 hours at 4°C. If a preservative pouch is added to the sample, stability of urine is extended to 2 days at room temperature or 4–6 days at 4°C.

This assay can detect both chlamydia and gonorrhoea and has an optional external amplification control available (*14*). The choice of tests requested is strip-specific (i.e. each strip of eight samples must be tested for the same combination of organisms) for chlamydia and gonorrhoea.

However, the use of amplification control is plate-specific such that if the option is chosen, it applies to all samples on that plate. The test is in a 96-well format with separate wells for each target sequence. Therefore, if requesting chlamydia, gonorrhoea, and amplification control, wells in each of 3 columns are used and a total of 32 samples and controls can be tested on one plate. Alternatively, if only chlamydia is requested, a total of 96 samples can be run on 1 plate.

Since the assay was designed to be a closed system, no enzymatic method to degrade carry-over contaminants from previous amplifications was included. Unfortunately, numerous incidents of gross environmental contamination have occurred. These events may take weeks of clean-up and recovery that can result in very costly delays and often require relocation to an area previously unexposed to the assay. Laboratories using this assay must adhere rigorously to an environmental monitoring plan to detect contamination events in a timely fashion. A second-generation assay that appears to be more resistant to this type of contamination is under evaluation.

BD also has developed a newer generation assay—the FDA-approved ProbeTec ET CTQx/GCQX on the Viper System with XTR (Viper). This assay also is fully automated and can generate 278 results during a single 8-hour work shift. This assay uses a duplex chemistry that includes an amplification detection paired with each chlamydial or gonococcal detection reaction. The system is highly robust with excellent time motion characteristics (*24*).

5.4.3 Gen-Probe diagnostic assays

The FDA-approved APTIMA Combo 2 assay (Gen-Probe, USA) is based on the principle of rRNA target capture (intended to reduce or eliminate inhibition of amplification), i.e. isolation of target rRNA sequences using capture oligonucleotides and DNA magnetic beads, followed by amplification using transcription-mediated amplification (TMA) technology of a sequence of the 23S rRNA of *C. trachomatis*. Amplification is detected using the kinetics of light emission from labelled DNA-probes complementary to the target region. Data confirming the lack of inhibition using this assay have been obtained primarily through use of negative patient samples spiked with laboratory strains of *C. trachomatis* (*25*).

Approved samples include urine, vaginal, endocervical, and urethral swabs and samples collected in liquid cytology medium (*12, 26*). Swabs are collected using the manufacturer's transport medium and are stable for up to 60 days at room temperature, making them ideal for transport to distant laboratories. First-catch urine is stable for up to 24 hours following collection and, once placed in the manufacturer's medium, is stable at room temperature for up to 30 days. The APTIMA Combo 2 test also detects *N. gonorrhoeae* (16S rRNA) and, in total, approximately 500 samples can be run during an 8-hour period using an automated system (the TIGRIS system). The popularity of this assay is rapidly increasing in many countries because of the extremely high sensitivity, specificity, extended sample stability, and the automation of the process.

Since samples remain sealed following amplification, the potential for carry-over environmental contamination is expected to be very low. However, environmental monitoring is strongly recommended in the absence of an enzymatic or other control measure for degradation of amplified product. The manufacturer strongly recommends stringent adherence to cleaning and decontamination procedures. Reproducibility should be monitored. Furthermore, for ideal specificity,

confirmatory assays detecting *C. trachomatis*-specific 16S rRNA (APTIMA CT) and *N. gonorrhoeae*-specific 16S rRNA (APTIMA GC; another sequence of 16S rRNA compared to the one used in APTIMA Combo 2) are available, and these assays are ideal for confirmation of positive results as well as for repeat testing (*26*).

5.4.4 Roche Diagnostics assays

The FDA-approved Cobas Amplicor CT/NG assay (Roche Diagnostics, USA) uses PCR technology to amplify target DNA sequences using organism-specific biotinylated primer pairs. The chlamydial target is located on the cryptic plasmid. Following a three-temperature amplification process, products are hybridized to magnetic beads coated with species-specific probe sequences located interior to the primer sequences. The detection process is based on biotin-avidin interactions. One Cobas instrument can test approximately 96 samples, including specimens and controls, in an 8-hour period. Swabs are collected in a commercially available transport medium. Urine and swabs are stable at 4°C for up to 7 days. Approved specimen types include endocervical swabs, urethral swabs, liquid cytology medium, and first-catch urine, although female urine should not be used for gonorrhoea testing. Vaginal swabs have demonstrated acceptable results with this platform (*27, 28*).

This assay includes a measure of inhibition based on an irrelevant DNA sequence pre-loaded into every reaction tube. This is intended to give the diagnostician evidence that a negative result is truly negative and not merely affected by contents of the sample. The assay also utilizes an enzyme (Uracile-N-glycosylase) in the amplification mix that degrades previously amplified sequences based on presence of dUTP rather than dTTP in the amplified product. This provides the user with a safety net to protect against minor splashes and aerosolization that routinely occur when handling a large number of samples and enhances reproducibility of results.

The Cobas TaqMan CT v2.0, with a CE mark in Europe, is the second-generation assay manufactured by Roche. This platform uses real-time PCR and can be coupled with an automated processing system to minimize technician hands-on time. The assay can be used for chlamydia alone without bundled gonorrhoea testing. The latest generation assay by Roche (Cobas 4800 CT/NG), which can also detect *N. gonorrhoeae*, is now FDA-approved. This assay uses real-time PCR technology on a fully automated platform. The primer targets used in both Cobas TaqMan CT v2.0 and Cobas 4800 CT/NG have been redesigned and include dual targets for chlamydia to ensure detection of the Swedish nvCT (*20–22*). The second target is not located on the plasmid but is located on conserved regions of the *ompA* gene encoding the major outer membrane protein (MOMP). The performance characteristics are in the range seen by other NAATs. This assay provides an amplification control and a carry-over contamination prevention process in conjunction with a truly walk-away automation system that can test up to 278 samples during an 8-hour shift. The assay works well with invasive samples as well as vaginal swabs for women and urine for men. It is under evaluation for liquid-based cytology medium.

5.4.5 Other NAAT assays

The assays described above are based on the information available at the time of preparation of this manual. Several new NAATs currently are under development and evaluation, and given the rapidly changing nature of this field, we can expect a proliferation of new tests. Laboratories must review the literature as descriptions of these new assays become available. Additionally, in many settings, FDA-approved assays are not readily available or cannot be performed due to the requirement for specialized instrumentation or high cost. As a result, commercially available assays that have not been reviewed by the FDA and laboratory-developed assays are used as methods that provide enhanced sensitivity compared to other classes of detection (e.g. DFA or ELISA). If non-FDA approved NAATs are used, regional (such as EU) and/or other national regulatory processes should provide safeguards on the quality and performance of the diagnostic NAAT (*17, 18*). It is in these cases also critical that laboratories engage in rigorous validation of the performance of these NAAT assays prior to using them for generation of patient results; see also Chapter 2 and Annex 3 regarding NAATs and their validation and QA.

5.5 Non-nucleic acid-based detection methods

5.5.1 Direct immunofluorescence assay (DFA)

DFA utilizes a fluorescein-tagged monoclonal antibody to allow microscopic visualization of *C. trachomatis* elementary bodies in cellular smears collected from conjunctiva, urethra, or the endocervix. This remains the only type of test that has the capacity to assess specimen quality directly; however, DFA suffers from suboptimal sensitivity even relative culture.

Two direct fluorescent antibody assays, MicroTrak direct stain (Trinity Biotech, Ireland) and Pathfinder direct stain (BioRad Laboratories, USA), are available for staining of smears collected from the urethra, endocervix, rectum, oropharynx, and conjunctiva, for the visualization of chlamydial elementary bodies. The MicroTrak assay uses a fluorescein-tagged monoclonal antibody specific for *C. trachomatis* MOMP and does not cross-react with *C. pneumoniae*.

In contrast, the Pathfinder reagent uses a polyclonal antibody specific for lipopolysaccharide (LPS) that is more broadly reactive and will stain *C. pneumoniae* as well as *C. trachomatis*. The most common use for DFA is staining of conjunctival smears, predominantly in neonates in developed countries. The DFAs offer the advantages of rapid turnaround time, high specificity, and detection of non-viable organisms. In addition, no other commercial assay has a regulatory claim for conjunctival sample or has the capacity to assess specimen quality directly. However, DFA suffers from substantially lower sensitivity than that of NAATs, is laborious and unsuitable for high-throughput diagnostics, and requires skilled microscopists. Negative results should be interpreted with caution due to the poor performance of these tests that may result in missed infections.

5.5.2 Point-of-care (POC) tests

ELISAs suffer from low sensitivity and suboptimal specificity (especially ELISAs detecting LPS) compared to NAATs, which may require confirmative testing of positive results. Negative results should be interpreted with caution due to the poor performance of these tests that may result in missed infections. Due to the limitation of existing ELISA, these tests should not be used when any other testing options are available.

However, unlike ELISA, POC tests ("rapid tests") that utilize ELISA technology have advantages that make their use reasonable in specific settings. Several POC tests, commonly based on lateral flow and antigen membrane capture on immunochromatographic strips (ICS), have been developed for diagnosis of *C. trachomatis* infections. Many of these tests have been developed and commercialized, but most have not undergone appropriate and comprehensive evaluation. However, when compared to NAATs, this rapid test displays clearly insufficient sensitivity, and it should only be used when adequate laboratory facilities are lacking. Annex 2 discusses the test principles for POC tests. Despite the low sensitivity of POC tests, in resource-constrained, high-prevalence populations, decreases in sensitivity may be acceptable in exchange for the ability to test and treat while the patient is on-site (*29*).

In a decision analysis study by Gift et al., when the patient return-for-treatment rate was 65% or lower, rapid POC diagnostics provided an increase in the number of patients treated even though fewer infections were identified (*30*). The POC tests could in these settings also be used to increase the specificity of the syndromic management algorithms, which will reduce overtreatment and screen for asymptomatic infections.

Rapid POC tests offer expanded opportunities for reaching non-clinic-based populations and providing immediate treatment. In some situations, the impact of this increased treatment may offset the lower sensitivity of these tests compared to NAATs.

> - Rapid POC tests offer expanded opportunities for reaching non-clinic-based populations and providing immediate treatment. In some situations, the impact of this increased treatment may offset the lower sensitivity of these tests compared to NAATs.

5.6 Methodologies for use in reference laboratories only

5.6.1 Culture

Until the early 1980s, the main and "gold standard" method for diagnosis of *C. trachomatis* infection was the centrifuge-assisted inoculation of clinical specimens onto susceptible viable cells in tissue culture, followed by the demonstration of characteristic chlamydial inclusions after incubation. Briefly, the specimen is collected using Dacron swabs or cytobrushes and placed into a transport medium, e.g. sucrose-phosphate-glutamate (SPG) buffer (see Annex 4) containing fetal bovine serum and antimicrobials such as vancomycin, gentamicin, and nystatin, to inhibit growth of other bacteria and fungi. While this method should now be reserved for use in reference laboratories, it is important to maintain the ability to obtain patient-derived isolates on occasion, and this requires use of tissue culture.

5.6.1.1   Culture of C. trachomatis *in McCoy cell line*

- Splitting flasks

  - Check media for sterility prior to use.

  - Check monolayers visually for confluency and lack of microbial contamination.

  - Proceed using sterile technique working in a biohazard containment hood. Aspirate media from confluent flask using a sterile pipette and a vacuum flask.

  - Rinse the monolayer with 10 ml glucose-potassium-sodium-phosphate (GKNP) solution (see Annex 4) and aspirate.

  - Add 4 ml trypsin and incubate cells at room temperature until the monolayer is loosened from the flask (approximately 3–7 minutes). Lightly tap the sides of the flask to remove the cells.

  - Add 4 ml of Iscove's modified Dulbecco medium (IMDM-VGA) (see Annex 4) to inactivate the trypsin and mix well. Use the liquid to rinse off any remaining cells on the back of the flask.

  - Add 1 ml of the cell suspension to each new 175-cm^2 flask and bring the total volume up to 75 ml with IMDM-VGA. When seeding a 75-cm^2 flask, add 0.5 ml and bring the total volume up to 35 ml.

  - Incubate flasks at 37°C for 48–96 hours with lids tightly capped.

- Seeding microtitre plates and vials

  - Follow steps 1–6 above.

  - Each plate to be seeded requires 15 ml of a diluted cell suspension. Calculate the total volume needed based on the number of plates desired. Example: 10 plates require 150 ml of IMDM-VGA. Each vial requires 1 ml of Modified Eagle's Medium (MEM-VG) (see Annex 4); calculate the total volume needed based on the number of vials desired.

  - For each 50 ml of IMDM-VGA for microtitre plates, add 1 ml of the cell suspension from the trypsinized flask. Example: 150 ml of IMDM-VGA needs 3 ml of cells. For each 100 ml of IMDM-VGA for vials, add 1 ml of the cells.

  - Load a hemacytometer with the diluted cell suspension. Count 5 squares (the 4 corners and the middle); the mean number of cells per square is multiplied by 10^5 to give the number of cells per millilitre. Microtitre plates require $1–1.4 \times 10^6$ cells/ml (10–14 cells per square); vials require $7–10 \times 10^5$ cells/ml in order to be confluent in 48 hours. If the number of cells counted is out of range, adjust the concentration by adding additional IMDM-VGA, or by adding more cells from the flask as appropriate. Re-count and adjust until achieving the correct concentration.

  - Add 200 µl to each well. Seal the plates with sealing film. Add 1 ml to each vial and cap tightly. Incubate at 37°C until use (48–96 hours).

- Inoculating microtitre plates

  - Check McCoy cell monolayer for confluency. Cells should be touching and slightly crowded. Media should be clear.

  - The following specimens should be tested in the microtitre format: cervical, urethral, vaginal, rectal, oropharyngeal, and conjunctival.

- Vortex specimens for 5 seconds then sonicate, if possible, for 20 seconds. During sonication, hearing and eye protection must be worn.

- Specimens that exhibit gross bacterial contamination (e.g. cloudy transport medium or pH change in medium) may cause toxicity in the tissue culture. These specimens should be tested undiluted and diluted at 1:2 and 1:10 (in SPG).

- Working in a biohazard containment hood, place one half of each specimen into the cryovial with the same specimen number.

- Aspirate plates containing confluent monolayers.

- Add 100 µl from each specimen and layer it onto the cell monolayer. Each specimen is inoculated onto three consecutive monolayers. After completing the inoculation of each plate, add 200 µl IMDM-VGA into each well.

- Seal plates with sealing film and centrifuge at 1400g for 1 hour at 30°C. Incubate at 37°C for 48 hours.

- Store specimens at –70°C until results are available. All positive specimens are stored >2 weeks.

- Remove media from plates. Fix monolayers by covering with methanol for 10 minutes. Remove the methanol.

- Inoculating vials

  - Only exceptional specimens are cultured in vials. Some examples include endometrial and tubal biopsies and lymph node tissue.

  - Check McCoy cell vials for confluency; cells should be touching and slightly elongated. Medium should be clear.

  - Vortex specimens for 5 seconds then sonicate for 20 seconds. During sonication, hearing and eye protection must be worn.

  - Specimens that exhibit gross bacterial contamination (e.g. cloudy transport medium or pH change in medium) may cause toxicity in the tissue culture. These specimens should be tested undiluted and diluted at 1:2 and 1:10 (in SPG).

- Working in the hood, label and aspirate three vials per specimen. Add 0.2 ml of specimen to each vial. Overlay with 1 ml of IMDM-VGA.

- Centrifuge at 2500g for 1 hour at 30°C. Incubate at 37°C for 72 hours.

- Store all specimens at –70°C until results are available. All positive specimens are stored indefinitely.

- Aspirate medium from 1 vial per specimen and fix by covering monolayer with methanol for 10 minutes.

- Staining with fluorescent antibodies

- Follow the package insert instructions for the specific anti-chlamydial antibody used. In general:

- Layer anti-serum on to fixed monolayer (after removal of methanol). Microtitre wells receive 40 µl each using a multi-channel pipettor.

- Incubate at room temperature for 30 minutes.

- Rinse gently three times with phosphate-buffered saline (PBS) solution (see Annex 4).

- Plates are read as is, 13 mm round coverslips are removed from the vials and, using mounting fluid, placed on glass slides with 22 × 50 mm coverslips. Keep stained cultures in the dark until they are read. Cultures should be read the same day they are stained.

In most laboratories, culture is no longer an appropriate diagnostic test since it is significantly less sensitive than NAATs, has a significantly longer turnaround time, demands invasive samples, requires more restrictive handling conditions to preserve the viability of *C. trachomatis*, is technically complex, and lacks internationally standardized and quality-assured methods. Culture is now requested predominantly for use in medico-legal cases, due to the assumption of 100% specificity (but cross-contamination between samples occurs), and for test-of-cure testing that is requested fewer than 14 days following treatment. In fact, the specificity of NAATs is such that these assays should be acceptable for medico-legal cases, and reliance on culture reflects the slow pace of change in legal

standards. Test-of-cure is rarely performed given the efficacy of single dose treatments now available; however, some recent studies have indicated suboptimal eradication efficacy of azithromycin 1 g. If performed, this is most often performed more than 2 to 3 weeks post-treatment at which point, in most cases, the NAAT assays are appropriate since DNA shedding from the initial infection should be complete by this time. However, the ideal time point for test-of-cure may differ for RNA-based NAATs.

Therefore, unless isolates of organisms are desirable for research purposes, there is no longer a valid justification for culture as a routine diagnostic method. This is consistent with the recommendations in the current CDC sexually transmitted diseases laboratory guidelines (15).

Isolates that are recovered in those settings in which culture is maintained should be preserved in a specimen repository for epidemiologic studies. These studies may include investigation of antimicrobial susceptibility (see below) and genotyping. Genotyping can be performed using molecular techniques and does not require viable organisms (31–33). Therefore, repositories of NAAT positive samples also should be maintained in regions throughout the world.

- Culture has a suboptimal sensitivity compared to commercially available and internationally approved NAATs and cannot be recommended for diagnostics if appropriate NAAT is available and affordable.
- Culture capability should be maintained in some reference laboratories and repositories of isolates should be stored for potential future phenotypic and/or genetic studies.

5.6.2 Antimicrobial susceptibility testing

There is no unambiguous evidence of emergence of acquired homotypic (phenotypically and genetically) and stable resistance to recommended antimicrobials in clinical *C. trachomatis* isolates, although case reports have suggested bacterial resistance as a cause of treatment failures. Furthermore, in vitro antimicrobial susceptibility testing of *C. trachomatis* has never been performed routinely, and a universally

accepted, standardized, reproducible, and quality-assured method as well as reliable and evidence-based correlates between in vitro activity and in vivo efficacy (clinical treatment outcome) are lacking (34).

The antimicrobial susceptibility testing of *C. trachomatis* is labour-intensive, requires tissue culture expertise, and is only feasible in reference laboratories. However, emergence and spread of clinically relevant antimicrobial resistance in *C. trachomatis* in the future should not be excluded. This highlights the need for appropriate evaluation of current antimicrobial susceptibility testing methods and development of an effective, standardized, objective, and quality-assured method as well as appropriate correlates between in vitro activity and treatment outcome. This method may be useful in the future for monitoring possible antimicrobial resistance, clinical treatment studies, and assessment of the in vitro activity of new antimicrobials. In the event of spread of clinically relevant antimicrobial resistance in *C. trachomatis*, repositories of isolates will be useful for retrospective evaluations in research settings.

5.6.3 Serology
Serological methods of diagnosing chlamydia infections were amongst the earliest techniques available. These methods identify and, in some cases, titrate the level of antibody response to chlamydial antigens. While the first assays, complement fixation and microimmunofluorescence (MIF), relied on whole organisms, subsequent assays were developed that are specific for responses to individual proteins or antibody class. Serology may aid in the diagnosis and/or screening for complicated *C. trachomatis* infections (reactive arthritis, PID, ectopic pregnancy, tubal factor infertility), be diagnostic in neonatal pneumonia and LGV infections (see Chapter 11), and be valuable in research and epidemiological studies, e.g. for the cumulative history of exposure of a sample population to chlamydial infection. Nevertheless, it is important to always interpret the serological results with caution and not out of context.

In an acute, primary chlamydial infection, specific IgM, but also IgG and IgA may be detected. However, systemic antibody response may be delayed or not

measurable after uncomplicated urogenital infection. In contrast, high levels of antibodies to *C. trachomatis* can persist long after infection has been cleared. Accordingly, due to the low sensitivity and specificity, measurement of chlamydial antibody has limited value for diagnosis of acute *C. trachomatis* infection and should not be used for routine diagnosis of uncomplicated *C. trachomatis* infections.

- Do not use serology for diagnosis of uncomplicated urogenital *C. trachomatis* infection.
- Only use serology as a possible aid in the diagnosis and/or screening for complicated *C. trachomatis* infections, neonatal pneumonia, and LGV infections, as well as in epidemiological studies.

5.7 References

1. Schachter J et al. Confirming positive results of nucleic acid amplification tests (NAATs) for *Chlamydia trachomatis*: all NAATs are not created equal. *Journal of Clinical Microbiology*, 2005, 43(3):1372–1373.

2. Martin DH et al. Use of multiple nucleic acid amplification tests to define the infected-patient "gold standard" in clinical trials of new diagnostic tests for *Chlamydia trachomatis* infections. *Journal of Clinical Microbiology*, 2004, 42(10):4749–4758.

3. Masek BJ et al. Performance of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by use of self-collected vaginal swabs obtained via an Internet-based screening program. *Journal of Clinical Microbiology*, 2009, 47(6):1663–1667.

4. Hobbs MM et al. From the NIH: proceedings of a workshop on the importance of self-obtained vaginal specimens for detection of sexually transmitted infections. *Sexually Transmitted Diseases*, 2008, 35(1):8–13.

5. Bachmann LH et al. Nucleic acid amplification tests for diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* rectal infections. *Journal of Clinical Microbiology*, 2010, 48(5):1827–1832.

6. Mimiaga MJ et al. Gonococcal, chlamydia, and syphilis infection positivity among MSM attending a large primary care clinic, Boston, 2003 to 2004. *Sexually Transmitted Diseases*, 2009, 36(8):507–511.

7. Annan NT et al. Rectal chlamydia—a reservoir of undiagnosed infection in men who have sex with men. *Sexually Transmitted Infections*, 2009, 85(3):176–179.

8. Schachter J et al. Nucleic acid amplification tests in the diagnosis of chlamydial and gonococcal infections of the oropharynx and rectum in men who have sex with men. *Sexually Transmitted Diseases*, 2008, 35(7):637–642.

9. Rosenberger JG et al. Reactions to self-sampling for ano-rectal sexually transmitted infections among men who have sex with men: a qualitative study. *Archives of Sexual Behavior*, 2011, 40(2):281–288.

10. Dodge B et al. Field collection of rectal samples for sexually transmitted infection diagnostics among men who have sex with men. *International Journal of STD and AIDS*, 2010, 21(4):260–264.

11. Black CM et al. Head-to-head multicenter comparison of DNA probe and nucleic acid amplification tests for *Chlamydia trachomatis* infection in women performed with an improved reference standard. *Journal of Clinical Microbiology*, 2002, 40(10):3757–3763.

12. Gaydos CA et al. Performance of the APTIMA Combo 2 assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in female urine and endocervical swab specimens. *Journal of Clinical Microbiology,* 2003, 41(1):304–309.

13. Horner P et al. Enhanced enzyme immunoassay with negative-gray-zone testing compared to a single nucleic acid amplification technique for community-based chlamydial screening of men. *Journal of Clinical Microbiology*, 2005, 43(5):2065–2069.

14. Van Der Pol B et al. Multicenter evaluation of the BDProbeTec ET System for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine specimens, female endocervical swabs, and male urethral swabs. *Journal of Clinical Microbiology*, 2001, 39(3):1008–1016.

15. Association of Public Health Laboratories (APHL). Laboratory diagnostic testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Expert consultation meeting summary report, 13–15 January 2009 Atlanta, GA. Silver Spring, MD, APHL, 2009 (http://www.aphl.org/aphlprograms/infectious/std/Documents/ID_2009Jan_CTGCLab-Guidelines-Meeting-Report.pdf).

16. Mahony J et al. Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition and removal of inhibitory activity. *Journal of Clinical Microbiology*, 1998, 36(11):3122–3126.

17. Unemo M et al. Can the Swedish new variant of *Chlamydia trachomatis* (nvCT) be detected by UK NEQAS participants from seventeen European countries and five additional countries/regions in 2009? *Eurosurveillance*, 2009, 14(19). pii:19206.

18. Reischl U, Straube E, Unemo M. The Swedish new variant of *Chlamydia trachomatis* (nvCT) remains

undetected by many European laboratories as revealed in the recent PCR/NAT ring trial organised by INSTAND e.V., Germany. *Eurosurveillance*, 2009, 14(32):pii:19302.

19. Marshall R et al., eds. New automated real time PCR technology for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. 11th International Symposium on Human Chlamydial Infections. Niagra-on-the-Lake, Ontario, 2006.

20. Unemo M et al. The Swedish new variant of *Chlamydia trachomatis*: genome sequence, morphology, cell tropism and phenotypic characterization. *Microbiology*, 2010, 156(Pt 5):1394–1404.

21. Ripa T, Nilsson P. A variant of *Chlamydia trachomatis* with deletion in cryptic plasmid: implications for use of PCR diagnostic tests. *Eurosurveillance*, 2006, 11(11):E061109.2.

22. Unemo M, Clarke IN. The Swedish new variant of *Chlamydia trachomatis*. *Current Opinion in Infectious Diseases*, 2011, 24(1):62–69.

23. Gaydos CA et al. Performance of the Abbott RealTime CT/NG for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *Journal of Clinical Microbiology,* 2010, 48(9):3236–3243.

24. Felder RA et al. Process evaluation of a fully automated molecular diagnostics system. *Journal of the Association for Laboratory Automation*, 2009, 14(5):262–268.

25. Chong S et al. Specimen processing and concentration of *Chlamydia trachomatis* added can influence false-negative rates in the LCx assay but not in the APTIMA Combo 2 assay when testing for inhibitors. *Journal of Clinical Microbiology,* 2003, 41(2):778–782.

26. Boyadzhyan B et al. Comparison of the APTIMA CT and GC assays with the APTIMA Combo 2 assay, the Abbott LCx assay, and direct fluorescent-antibody and culture assays for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *Journal of Clinical Microbiology,* 2004, 42(7):3089–3093.

27. Schachter J et al. Vaginal swabs are appropriate specimens for diagnosis of genital tract infection with *Chlamydia trachomatis*. *Journal of Clinical Microbiology,* 2003, 41(8):3784–3789.

28. Gaydos CA et al. Evaluation of dry and wet transported intravaginal swabs in detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in female soldiers by PCR. *Journal of Clinical Microbiology,* 2002, 40(3):758–761.

29. Huppert J, Hesse E, Gaydos CA. What's the point? How point-of-care STI tests can impact infected patients. *Point of Care*, 2010, 9(1):36–46.

30. Gift TL et al. The rapid test paradox: when fewer cases detected lead to more cases treated: a decision analysis of tests for *Chlamydia trachomatis*. *Sexually Transmitted Diseases*, 1999, 26(4):232–240.

31. Bandea CI et al. *Chlamydia trachomatis* serovars among strains isolated from members of rural indigenous communities and urban populations in Australia. *Journal of Clinical Microbiology*, 2008, 46(1):355–356.

32. Lima HE et al. Genotyping of *Chlamydia trachomatis* from endocervical specimens in Brazil. *Sexually Transmitted Diseases*, 2007, 34(9):709–717.

33. Pedersen LN, Herrmann B, Møller JK. Typing *Chlamydia trachomatis*: from egg yolk to nanotechnology. *FEMS Immunology and Medical Microbiology*, 2009, 55(2):120–130.

34. Wang SA et al. Evaluation of antimicrobial resistance and treatment failures for *Chlamydia trachomatis*: a meeting report. *Journal of Infectious Diseases,* 2005, 191(6):917–923.

Chapter 6

Trichomoniasis

6.1 Introduction

Trichomonas vaginalis is the etiological agent of the most prevalent non-viral sexually transmitted infection (STI) worldwide. In 2008, the World Health Organization estimated 276.4 million new cases of *T. vaginalis* to have occurred globally among adults ages 15–49 years. This represented substantially more STI cases than those caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* combined (*1*). Despite this high prevalence of infection with *T. vaginalis*, STI control efforts have historically underemphasized this pathogen. Although *T. vaginalis* may cause an abnormal vaginal discharge (trichomoniasis) in women and may be responsible for as much as 10–12% of non-gonococcal urethritis cases in men (*2*), the infection may be asymptomatic in at least 50% of women and 70–80% of men (*3*). Thus, laboratory diagnosis is essential for supplementing syndromic management strategies for the treatment of this infection.

T. vaginalis is a motile, ovoid, pear-shaped, flagellated protozoan (10–20 μm long). The organism has four free, anterior flagella and a fifth flagellum embedded in an undulating membrane that extends around the anterior two thirds of the cell. The flagella move the protozoan with a jerky movement.

The epidemiology of *T. vaginalis* infection differs from other infections that cause a genital discharge in two important aspects. First, the age distribution of infection is distinct from that of chlamydial infection or gonorrhoea in that chlamydial, and to a lesser extent gonococcal, prevalence rates peak in women ages 15–25 years, whereas *T. vaginalis* infections appear to peak substantially later in life (between 40–50 years of age) (*4*, *5*). This difference in distribution is relevant to informing STI control programmes and appropriate targeting of screening efforts. The age-specific

distribution of infection in men has not been studied adequately.

The second difference is that, despite reliance on sexual transmission for movement from host to host, the gender distribution of laboratory-diagnosed *T. vaginalis* infections is highly skewed with a female-to-male ratio as high as 4:1 (*6*–*8*). This distribution is demonstrated in the infection rates of male partners to infected women, which ranges from 22% to 72% (*9*) and the few studies done in both men and women. This is likely due to a more transient infection occurring in men, brief window of opportunity for detection of the organism, and lack of screening and diagnosis in men. *T. vaginalis* adheres to the mucous membranes associated with squamous epithelium and does not invade the mucosa. Because of the environment of the male urethra, this organism is less likely to be maintained in that location than in the vaginal milieu. There are limited data regarding the pathogenesis of *T. vaginalis* infection in men (*8*). In men and women, the organism often elicits a robust inflammatory response that results in the fulminate discharge of overt disease. See Table 6.1 for a description of the clinical manifestations of *T. vaginalis* infection.

The diagnosis of trichomoniasis is based on the odour, quality, and quantity of vaginal discharge; the vaginal pH; and possible presence of cervical friability. Vaginal pH, usually >6.0, fulminate or frothy white discharge, and punctate cervical friability ("strawberry cervix") are suggestive of infection with *T. vaginalis*. However, the absence of these clinical signs is not sufficient evidence to rule out infection, especially when the infection is asymptomatic in nearly 50% of women and 70–80% of men (*5*, *6*). Accordingly, to enhance the sensitivity and specificity of diagnosis, laboratory methods are required.

Table 6.1: Clinical manifestations of _Trichomonas vaginalis_ infection

Genital infection	Primary	Sequelae
Women	Fulminate, purulent, or frothy white to yellow discharge, dysuria, pelvic pain, itching	Adverse outcomes of pregnancy, increased risk of HIV transmission and acquisition
Men	Urethral discharge, dysuria, testicular pain	Possible epidydimitis and prostatitis

The inflammatory response in trichomoniasis in women and _T. vaginalis_ urethritis in men is significant and substantially increases the risk of HIV transmission and acquisition, as well as increase the probability of adverse outcomes of pregnancy. In men, treatment of _T. vaginalis_ urethritis results in a 0.5–2 log decrease in the seminal fluid HIV viral load (_10_, _11_). Similar results have been observed in women treated for vaginal discharge (_12_, _13_). Given that genital compartment viral load is one of the most significant risk factors for transmission to an uninfected partner, appropriate diagnosis and treatment of _T. vaginalis_ infections should be a public health priority as an HIV reduction strategy. Similarly, data have been available for many years indicating that the presence of discharge-causing STI increases the risk of HIV acquisition. Studies performed in sub-Saharan Africa have estimated the increased risk of HIV seroconversion in women with trichomoniasis to be 1.5- to 3.0-fold (_9_). These studies have been conducted in populations of women at high risk for HIV (female sex workers) and more general populations (women attending family planning clinics). Survival analysis performed during a large clinic trial estimated a twofold increase in relative risk for HIV infection throughout a 30-month period for women with trichomoniasis (_14_). Conversely, women with HIV were at a 2.1-fold increase in risk of acquiring trichomonas during the 30 months of follow-up (_14_). This indicates an increased risk of transmission to HIV-uninfected partners. With increased risks of enhanced HIV transmission similar to those seen with gonorrhoea, and prevalence rates that are nearly three-fold higher, appropriate diagnosis and treatment of _T. vaginalis_ infection deserve increased prioritization.

- _T. vaginalis_ infections are the most common non-viral STI and enhance HIV transmission and acquisition, and increase the probability of adverse outcomes of pregnancy.

6.2 Overview of available diagnostic methods

There are four main classes of laboratory diagnostic assays: wet preparation microscopy, antigen detection, culture, and nucleic acid amplification tests (NAATs). Wet preparation microscopy may be performed in clinical settings and in combination with testing for bacterial vaginosis. This is an ideal first-line diagnostic method, i.e. if positive, it provides a definitive diagnosis with high specificity if adequately performed and interpreted. However, use caution in ruling out infection based solely on negative microscopy for three reasons. First, trichomonads are highly temperature-sensitive and lose their motility in as few as 10 minutes following sample collection. Since motility is a hallmark feature, this loss may result in false-negative results. Second, the size of trichomonads is similar to that of white blood cells (lymphocytes or small neutrophil granulocytes), which are often present as a result of the inflammatory process. Thus, the trichomonads may be obscured by or mistaken for these cells. Finally, in many women and in most men, the organism load may be below the limit of detection for microscopy. In addition to vaginal swab samples, microscopy may be performed on urethral exudates or urine sediment from men, but this technique suffers from low sensitivity, probably also due to low organism load (_15_).

Point-of-care (POC) antigen detection tests are now available in many settings. These are approved only for female vaginal swab samples. The latest generation of these tests, e.g. OSOM Trichomonas Rapid Test (Genzyme Diagnostics, USA), has superior sensitivity compared to microscopy (*16*, *17*) and can provide results in approximately 30 minutes, i.e. while the patient waits. As with other POC assays, the opportunity to treat infections immediately is an advantage of this test over tests that need referral to a central laboratory.

Laboratory-based culture has been available for many years and in the last decade, commercially available culture kits, such as InPouch TV culture system (BioMed Diagnostics, USA), have become available. Vaginal swabs, urethral swabs, and urine sediment from men are specimens licensed for culture (see Table 6.2). This method requires up to 5–7 days post-collection, and determination of positive results requires microscopy. However, culture increases the sensitivity beyond that of wet preparation microscopy (*15–17*).

Finally, NAATs are available for detection of specific *T. vaginalis* DNA or RNA. For programmes that employ NAATs for chlamydia and gonorrhoea, inclusion of *T. vaginalis* testing may be a reasonable strategy. At the time of this writing, the United States of America Food and Drug Administration (FDA) has approved only one assay, but other assays are under evaluation and may already be available in some settings. The sensitivity and specificity of the FDA-approved assay (APTIMA TV, Gen-Probe, USA) are very high (*5*, *16*, *18*). The performance characteristics of other NAATs must be rigorously evaluated, ideally to the FDA-approved test, in the setting for use and prior to implementation for routine diagnostics in the laboratory.

A number of methods have been described for the detection of antibodies to *T. vaginalis*. However, antibody tests have a low sensitivity and suboptimal specificity for the detection of current *T. vaginalis* infection, and should not be used for routine diagnosis of trichomoniasis.

Table 6.3 summarizes the performance characteristics of available diagnostic tests for detection of *T. vaginalis*. For appropriate performance of all diagnostic methods, it is crucial to follow precisely the standard operating procedures and recommendations from the manufacturer concerning collection, transportation, and storage of samples, as well as performance of the specific assay, including quality controls.

> - Appropriate POC tests and culture have higher sensitivity than microscopy.
> - Appropriate and validated NAATs have superior sensitivity relative to other diagnostic methods.

6.3 Collection, transportation, and storage conditions of specimens (see Table 6.2)

Women

Vaginal samples are optimal for detection of *T. vaginalis*. Sampling of the posterior fornix should be done using a Dacron or rayon swab on a plastic shaft. Cotton swabs on wooden shafts are acceptable for microscopy or culture inoculation, but are not recommended for POC or NAAT. Therefore, to avoid confusion, it is practical to avoid use of cotton swabs. Clinicians may collect samples prior to insertion of a speculum during pelvic examinations, or patients may self-obtain samples. Clinicians should provide instructions to ensure that patients understand how to collect their own samples. Provision of appropriate instructions has been found to be the determining factor in patient acceptability of self-sampling. Residual samples—vaginal, cervical, or urine—collected for chlamydia/gonorrhoea testing may be used for *T. vaginalis* NAAT.

Men

Urethral exudates may be collected using a Dacron or rayon swab on an aluminium shaft. These samples may be used for wet preparation microscopy, culture, or NAAT. First-void urine may be centrifuged to obtain sediment that is appropriate for culture. Non-centrifuged urine is adequate for NAAT testing.

Table 6.2: Sample collection, transportation, and storage

Site	Collection device	Sampling procedure	Microscopy	POC	Culture	NAAT
Vaginal (clinician-collected)	Swab/plastic[a]	Sample the posterior fornix prior to insertion of speculum.	Elute in ≤0.5 ml saline, place 1 drop of saline on slide with coverslip.	Place into kit extraction buffer and follow package insert directions.	Place directly into culture medium (e.g. Diamonds or InPouch).[b] Incubate at 37°C.	Place into manufacturer's collection device. Can be sent dry to laboratory. Store and transport according to package insert directions.
Vaginal (patient-collected)	Swab/plastic[a]	Rotate the swab 360°, touching all of the vaginal walls.	Hand to provider for elution and slide preparation.	Hand to provider for processing as above.	Hand to provider for inoculation into culture medium.	Place into manufacturer's collection device. Can be sent dry to laboratory. Store and transport according to instructions.
Urethra (should be used only with symptomatic men)	Swab/aluminium[c]	Collect urethral exudates >1 hour after previous void.	Elute in ≤0.5 ml saline, place 1 drop of saline on slide with coverslip.	NA	Place directly into culture medium (e.g. Diamonds or InPouch).[b] Incubate at 37°C.	Place into manufacturer's collection device. Store and transport according to package insert directions.
Urine	Sterile urine cup	Do not have patient clean the genital area. Obtain first portion of the void (in general less than 25 ml), >1 hour after previous void.	Centrifuge at 500g for 5 min. Place sediment into ≤0.5 ml saline. Place one drop of saline on slide with coverslip.	NA	Centrifuge at 500g for 5 min. Place sediment into culture medium (e.g. Diamonds or InPouch).[b] Incubate at 37°C.	Place into manufacturer's collection device. Store and transport according to package insert directions.

NA, not applicable; NAAT, nucleic acid amplification test; POC, point-of-care.

[a] Dacron or rayon swabs on a plastic shaft.

[b] For sites that do not have access to culture medium, swabs can be placed in tubes containing Amies medium and stored at 4°C for transportation to arrive in the central laboratory within 24 hours.

[c] Dacron or rayon swabs on an aluminium shaft.

Table 6.3: Performance characteristics of diagnostic tests for detection of *T. vaginalis*

	Microscopy	POC	Culture	NAAT
Specimen types				
Endocervical swab	No	No	No	Yes
Liquid cytology medium	No	No	No	Yes
Vaginal swabs				
Self-obtained	Yes	Yes	Yes	Yes
Clinician-collected	Yes	Yes	Yes	Yes
Urine				
Female	No	No	No	Yes
Male	Yes	No	Yes	Yes
Male urethral swab	Yes	No	Yes	Yes
Performance				
Sensitivity	Low	High[a]	Moderate–high	Very high
Specificity	Very high	Very high	Very high	Very high
Other considerations				
Cost	Low	Moderate	Moderate	High
Transportation and storage	NA	NA	Ambient	Ambient
Instrumentation	Microscope	None	Incubator, microscope	Large footprint
Throughput/ Automation	Low/no	Low/no	Low/no	High/possible
Technical complexity	Moderate (microscopy skills)	Low	Moderate (microscopy skills)	High
Level of laboratory infrastructure	Peripheral	Peripheral	Intermediate–central	Central
Other comments	• False-negative results are more likely than real negative results so clinical context is critical.	• Infections identified may be treated before the patient leaves the clinic.	• Strict attention to accurate microscopy is required. • Potential to obtain viable isolates is useful for additional testing such as genotyping and antimicrobial susceptibility testing.	• Potential for laboratory contamination requires strict adherence to protocols. • Some require large batch size, which may delay turnaround time.

NA, not applicable; NAAT, nucleic acid amplification test; POC, point-of-care.

[a] Refers to OSOM Trichomonas Rapid Test (Genzyme Diagnostics, USA).

6.4 Diagnostic Methods

6.4.1 Microscopy

Immediately following collection, swabs should be eluted into ≤0.5 ml of sterile saline at room temperature and a slide should be prepared with a drop of the saline sample and a coverslip. The slide should be read at 100× magnification within 10 minutes of collection to look for motile trichomonads. Confirmation of pear-shaped morphology, including visualization of flagella, should be performed using 400× magnification (Fig. 6.1). Non-motile cells cannot be diagnosed as trichomonads and, accordingly, immediate microscopy is crucial because the trichomonads quickly lose their motility (*19*). The sensitivity of microscopy is limited (as low as 40–65% for women in some settings and even lower for samples from men) (*6*, *15*, *16*) and negative results should be interpreted with caution.

In some settings, microscopy is the first-line diagnosis and screening, and negative samples are referred to a central laboratory for further evaluation, particularly in symptomatic individuals. However, when the strictly required motility and the morphology are identified, the specificity of microscopy is excellent and all patients with positive microscopy results should be considered infected.

Microscopy must be performed and interpreted within 10 minutes for optimal results, and has highest sensitivity in symptomatic women.
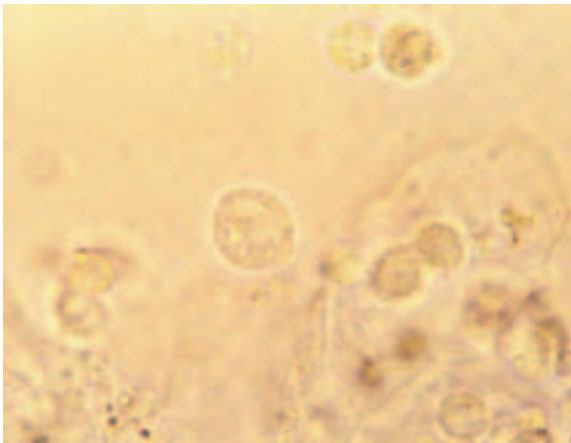
- Microscopy must be performed and interpreted within 10 minutes for optimal results, and has highest sensitivity in symptomatic women.

6.4.2 Point-of-care (POC) antigen detection tests

Several antigen detection assays have been developed for detection of *T. vaginalis* (*16*, *17*, *20*). Requirements for equipment and costs of reagents vary as does assay performance. Several of these assays are intended for use only in symptomatic women, making them less useful than other options. The latest generation of these tests, e.g. OSOM Trichomonas Rapid Test (Genzyme Diagnostics, USA), has superior sensitivity compared to microscopy (*16*, *17*). Procedures will vary by manufacturer and the package insert directions should be followed precisely for each specific assay.

Appropriate POC tests have substantially higher sensitivity than microscopy and provide rapid results with minimal technical expertise.

- Appropriate POC tests have substantially higher sensitivity than microscopy and provide rapid results with minimal technical expertise.

6.4.3 Culture

Culture has been the cornerstone for *T. vaginalis* diagnosis for many years. *T. vaginalis* is an anaerobic organism that grows more slowly under aerobic conditions. Nowadays, culture is usually performed using modified Diamond's medium or the commercial InPouch TV culture system (BioMed Diagnostics, USA) (Fig. 6.2). Cultures should be incubated for up to 5–7 days. It is important to note that *T. vaginalis* should be grown at the bottom of the culture tube or pouch and, accordingly, the tube or tubes should be incubated in a vertical position. Furthermore, culture medium should be pre-reduced and culture tubes slightly opened before placement in an anaerobic jar for incubation at 37°C.

Diamond's original medium (*21*, *22*) (see Annex 4) has subsequently been modified according to Fouts and

Figure 6.1
Trichomonas vaginalis **in wet preparation microscopy**

Kraus, i.e. with streptomycin replaced by netilmicin (*22, 23*). The modified medium improves the sensitivity to approximately 75% relative to NAATs. However, culture of *T. vaginalis* has disadvantages as well; the sensitivity of culture is low compared to results obtained with NAAT (especially for men), it is a time-consuming procedure, and it requires that patients return for results. Other culture media have also been described (*22, 24–26*) (see Annex 4). Swabs should be eluted or urine sediment inoculated directly into culture medium at the clinical site. For sites that do not have access to culture medium due to short shelf-life or other reasons, swabs can be placed in tubes containing Amies medium and stored at 4°C for transportation to arrive at the central laboratory within 24 hours. Cultures should be gently mixed and a drop taken from the bottom of the tube (highest concentration of trichomonads) for wet preparation microscopy each day for up to 7 days. If examination is not possible to perform daily, examination after 3–4 days and again at 7 days will detect almost all positive specimens.

In the mid-1990s, a culture system became commercially available that increased the use of culture for diagnosis (*22, 27–30*). This system, the InPouch TV culture system, utilizes a self-contained two-chamber pouch (Fig. 6.2). Swab samples are eluted or urine sediment is inoculated into the medium in the first section, the medium is forced into the second section, and the pouch is sealed. Pouches are transferred to the laboratory for

Figure 6.2
InPouch TV culture system

incubation at 37°C for up to 5 days. The pouch system can be concluded earlier than standard culture since the entire volume is assessed each time. Sealed pouches are read by placing the entire pouch on the microscope stage, using 100× magnification. Pouch holders that fit into the slide clips are available and should be used to assist with movement of the pouch during microscopy. The entire culture is read by carefully scanning from end to end. It is important to note that the thickness of the pouch requires that all planes of focus be evaluated; this requires movement top to bottom as well as side to side. Slides should be read daily until day 5. If no trichomonads are identified by day 5, the culture is reported as negative.

- Culture has sensitivity similar to appropriate POC test, but may be used also for testing men.

6.4.4 Nucleic acid amplification tests (NAATs)

NAATs offer the greatest flexibility in sample collection methods as well as the highest sensitivity of all available diagnostic methods. Residual genital samples used for diagnosis of chlamydia and gonorrhoea using NAATs are appropriate for detection of *T. vaginalis* nucleic acids. Laboratories that routinely run chlamydia and gonorrhoea NAATs should consider testing for trichomonas. Despite the difference in age-specific prevalence, in many settings the population prevalence of *T. vaginalis* infection is sufficient to warrant inclusion as part of a discharge-causing panel of tests. Descriptions of laboratory-developed assays that utilize many of the currently available chlamydia and gonorrhoea NAAT platforms are available (*6, 15, 31–42*). All of these assays involve processing to isolate nucleic acids, amplification of target sequences, and detection of amplified material. In addition to excellent sensitivity and specificity, these assays may be affected by environmental contamination and thus warrant strict adherence to good laboratory practice. It is also strongly recommended that the effectiveness of the proposed *T. vaginalis* NAAT for the local settings, before use in diagnostics, is strictly validated against at least one internationally validated NAAT, ideally the FDA-approved APTIMA TV (see below), and subsequently used with

appropriate quality assurance system (see Chapter 2 and Annex 3).

As aforementioned, one assay (APTIMA TV, Gen-Probe, USA) has FDA approval. This test has proven to be highly sensitive and specific (*16*, *18*, *43*, *44*), and can be run on a semi-automated or on a fully automated platform. The assay, like all NAATs, requires careful attention to liquid handling procedures to minimize contamination events. The equipment footprint and power requirements for this test are both substantial. This assay should be restricted to reference laboratories with high technical capacity.

> - Effective and validated NAATs have very high sensitivity and are especially useful in settings where chlamydia/gonorrhoea testing is being performed using NAATs.
> - NAAT diagnostics requires specialized equipment, reagents, and technical expertise.

6.5 Antimicrobial resistance

Isolates of *T. vaginalis* with decreased susceptibility and resistance to metronidazole (in general first-line treatment) and tinidazole (usually second-line therapy) have been described, but occur rarely (*45*, *46*). Nevertheless, in certain settings low-level metronidazole resistance has been identified in 2–5% of trichomoniasis cases in women. The origin of the antimicrobial resistance is unclear, but many of the resistant *T. vaginalis* strains appear to be aero-tolerant. Organisms that are facultative are generally resistant to metronidazole. However, the resistance data are scant and additional studies in this area are needed to determine the mechanisms, prevalence, and epidemiology of this resistance. However, as most organisms are susceptible, antimicrobial susceptibility testing (*47*, *48*) is not routinely performed. Nevertheless, some reference laboratories need to maintain capacity to perform antimicrobial susceptibility testing of *T. vaginalis* isolates, particularly when patients appear to fail metronidazole therapy. Patients returning with continued symptoms should be evaluated in the context of possible behavioral exposures and adherence to treatment regimens.

> - Clinicians should be aware that antimicrobial resistance has been described and consider this issue for people with continuing symptoms.

6.6 References

1. *Global incidence and prevalence of selected curable sexually transmitted infections—2008*. Geneva, World Health Organization, 2012 (http://www.who.int/reproductivehealth/publications/rtis/2008_STI_estimates.pdf, accessed 2 April 2013).

2. Wetmore CM, Manhart LE, Golden MR. Idiopathic urethritis in young men in the United States: prevalence and comparison to infections with known sexually transmitted pathogens. *Journal of Adolescent Health*, 2009, 45(5):463–472.

3. Seña AC et al. *Trichomonas vaginalis* infection in male sexual partners: implications for diagnosis, treatment, and prevention. *Clinical Infectious Diseases*, 2007, 44(1):13–22.

4. Zhang ZF et al. *Trichomonas vaginalis* and cervical cancer. A prospective study in China. *Annals of Epidemiology*, 1995, 5(4):325–332.

5. Van Der Pol B. *Trichomonas vaginalis* infections. In: Kumar B, Gupta S, eds. *Sexually transmitted infections*, 2nd ed. New Delhi, Elsevier, 2012:602–609.

6. Van Der Pol B, Kraft CS, Williams JA. Use of an adaptation of a commercially available PCR assay aimed at diagnosis of chlamydia and gonorrhea to detect *Trichomonas vaginalis* in urogenital specimens. *Journal of Clinical Microbiology,* 2006, 44(2):366–373.

7. Miller WC et al. The prevalence of trichomoniasis in young adults in the United States. *Sexually Transmitted Diseases*, 2005, 32(10):593–598.

8. Krieger JN. Trichomoniasis in men: old issues and new data. *Sexually Transmitted Diseases*, 1995, 22(2):83–96.

9. Shafir SC, Sorvillo FJ, Smith L. Current issues and considerations regarding trichomoniasis and human immunodeficiency virus in African-Americans. *Clinical Microbiology Reviews*, 2009, 22(1):37–45.

10. Price MA et al. Addition of treatment for trichomoniasis to syndromic management of urethritis in Malawi: a randomized clinical trial. *Sexually Transmitted Diseases*, 2003, 30(6):516–522.

11. Hobbs MM et al. *Trichomonas vaginalis* as a cause of urethritis in Malawian men. *Sexually Transmitted Diseases*, 1999, 26(7):381–387.

12. Tanton C et al. Correlates of HIV-1 genital shedding in Tanzanian women. *PloS One*, 2011, 6(3):e17480.

13. Wang CC et al. The effect of treatment of vaginal infections on shedding of human immunodeficiency virus type 1. *Journal of Infectious Diseases*, 2001, 183(7):1017–1022.

14. Mavedzenge SN et al. Epidemiological synergy of *Trichomonas vaginalis* and HIV in Zimbabwean and South African Women. *Sexually Transmitted Diseases*, 2010, 37(7):460–466.

15. Hobbs MM et al. Methods for detection of *Trichomonas vaginalis* in the male partners of infected women: implications for control of trichomoniasis. *Journal of Clinical Microbiology*, 2006, 44(11):3994–3999.

16. Huppert JS et al. Rapid antigen testing compares favorably with transcription-mediated amplification assay for the detection of *Trichomonas vaginalis* in young women. *Clinical Infectious Diseases*, 2007, 45(2):194–198.

17. Huppert JS et al. Use of an immunochromatographic assay for rapid detection of *Trichomonas vaginalis* in vaginal specimens. *Journal* of *Clinical Microbiology*, 2005, 43(2):684–687.

18. Nye MB, Schwebke JR, Body BA. Comparison of APTIMA *Trichomonas vaginalis* transcription-mediated amplification to wet mount microscopy, culture, and polymerase chain reaction for diagnosis of trichomoniasis in men and women. *American Journal of Obstetrics and Gynecology*, 2009, 200(2):188.e1–188.e7.

19. Kingston MA, Bansal D, Carlin EM. "Shelf life" of *Trichomonas vaginalis*. *International Journal of STD and AIDS*, 2003, 14(1):28–29.

20. Adu–Sarkodi Y et al. Comparison of latex agglutination, wet preparation, and culture for the detection of *Trichomonas vaginalis*. *Sexually Transmitted Infections*, 2004, 80(3):201–203.

21. Diamond LS. The establishment of various trichomonads of animals and man in axenic cultures. *Journal of Parasitology*, 1957, 43(4):488–490.

22. Schmid GP et al. Evaluation of six media for the growth of *Trichomonas vaginalis* from vaginal secretions. *Journal of Clinical Microbiology*, 1989, 27(6):1230–1233.

23. Fouts AC, Kraus SJ. *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. *Journal of Infectious Diseases*, 1980, 141(2):137–143.

24. Kupferberg AB et al. Nutritional requirements of *Trichomonas vaginalis*. *Proceedings of the Society for Experimental Biology and Medicine*, 1948, 67(3):304–308.

25. Kupferberg AB. *Trichomonas vaginalis*: nutritional requirements and diagnostic procedures. *International Record of Medicine and General Practice Clinics*, 1955, 168(11):709–717.

26. Feinberg JG, Whittington MJ. A culture medium for *Trichomonas vaginalis donné* and species of Candida. *Journal of Clinical Pathology*, 1957, 10(4):327–329.

27. Borchardt KA et al. A comparison of the sensitivity of the InPouch TV, Diamond's and Trichosel media for detection of *Trichomonas vaginalis*. *Genitourinary Medicine*, 1997, 73(4):297–298.

28. Borchardt KA. Trichomoniasis: its clinical significance and diagnostic challenges. *American Clinical Laboratory*, 1994, 13(9):20–21.

29. el Naga IF, Khalifa AM, el Azzouni MZ. In-pouch TV culture system in diagnosis of *Trichomonas vaginalis* infection. *Journal of the Egyptian Society of Parasitology*, 2001, 31(3):647–656.

30. Levi MH et al. Comparison of the InPouch TV culture system and Diamond's modified medium for detection of *Trichomonas vaginalis*. *Journal of Clinical Microbiology*, 1997, 35(12):3308–3310.

31. Smith KS et al. Comparison of conventional testing to polymerase chain reaction in detection of *Trichomonas vaginalis* in indigenous women living in remote areas. *International Journal of STD and AIDS*, 2005, 16(12):811–815.

32. Wendel KA et al. Use of urine polymerase chain reaction to define the prevalence and clinical presentation of *Trichomonas vaginalis* in men attending an STD clinic. *Sexually Transmitted Infections*, 2003, 79(2):151–153.

33. Kaydos-Daniels SC et al. Validation of a urine-based PCR-enzyme-linked immunosorbent assay for use in clinical research settings to detect *Trichomonas vaginalis* in men. *Journal of Clinical Microbiology*, 2003, 41(1):318–323.

34. Schwebke JR, Lawing LF. Improved detection by DNA amplification of *Trichomonas vaginalis* in males. *Journal of Clinical Microbiology*, 2002, 40(10):3681–3683.

35. Kaydos SC et al. Development and validation of a PCR-based enzyme-linked immunosorbent assay with urine for use in clinical research settings to detect *Trichomonas vaginalis* in women. *Journal of Clinical Microbiology*, 2002, 40(1):89–95.

36. Jordan JA, Lowery D, Trucco M. TaqMan-based detection of *Trichomonas vaginalis* DNA from female genital specimens. *Journal of Clinical Microbiology*, 2001, 39(11):3819–3822.

37. Lawing LF, Hedges SR, Schwebke JR. Detection of trichomonosis in vaginal and urine specimens from women by culture and PCR. *Journal of Clinical Microbiology*, 2000, 38(10):3585–3588.

38. Crucitti T et al. Comparison of culture and different PCR assays for detection of *Trichomonas vaginalis* in self collected vaginal swab specimens. *Sexually Transmitted Infections*, 2003, 79(5):393–398.

39. Pillay A et al. Comparison of a TaqMan-based real-time polymerase chain reaction with conventional tests for the detection of *Trichomonas vaginalis*. *Sexually Transmitted Infections*, 2007, 83(2):126–129.

40. Schirm J et al. *Trichomonas vaginalis* detection using real-time TaqMan PCR. *Journal of Microbiological Methods*, 2007, 68(2):243–247.

41. Simpson P et al. Real-time PCRs for detection of *Trichomonas vaginalis* beta-tubulin and 18S rRNA genes in female genital specimens. *Journal of Medical Microbiology*, 2007, 56(Pt 6):772–777.

42. Shipitsyna E et al. Evaluation of polymerase chain reaction assays for the diagnosis of *Trichomonas vaginalis* infection in Russia. *Journal of the European Academy of Dermatology and Venereology*, 2013, 27(2):e217–223.

43. Hardick A et al. Comparison between the Gen-Probe transcription-mediated amplification *Trichomonas vaginalis* research assay and real-time PCR for *Trichomonas vaginalis* detection using a Roche LightCycler instrument with female self-obtained vaginal swab samples and male urine samples. *Journal of Clinical Microbiology*, 2006, 44(11):4197–4199.

44. Munson E et al. Impact of *Trichomonas vaginalis* transcription-mediated amplification-based analyte-specific reagent testing in a metropolitan setting of high sexually transmitted disease prevalence. *Journal of Clinical Microbiology*, 2008, 46(10):3368–3374.

45. Sobel JD, Nyirjesy P, Brown W. Tinidazole therapy for metronidazole-resistant vaginal trichomoniasis. *Clinical Infectious Diseases*, 2001, 33(8):1341–1346.

46. Schmid G et al. Prevalence of metronidazole-resistant *Trichomonas vaginalis* in a gynecology clinic. *Journal of Reproductive Medicine*, 2001, 46(6):545–549.

47. Upcroft JA, Upcroft P. Drug susceptibility testing of anaerobic protozoa. *Antimicrobial Agents and Chemotherapy*, 2001, 45(6):1810–1814.

48. Meri T et al. Resistance of *Trichomonas vaginalis* to metronidazole: report of the first three cases from Finland and optimization of in vitro susceptibility testing under various oxygen concentrations. *Journal of Clinical Microbiology*, 2000, 38(2):763–767.

Chapter 7

Bacterial vaginosis

7.1 Introduction

Bacterial vaginosis (BV) is the most common cause of vaginal discharge among women of childbearing age. Although not considered a sexually transmitted disease, sexual activity is a risk factor for its acquisition (*1*). This has been shown by increases in its incidence with an increase in the number of recent and lifetime partners, and having a new sexual partner. The finding of bacterial vaginosis also in virginal females precludes its exclusive sexual transmission. The condition is probably much more related to alterations in the vaginal ecology (due to so-far-unknown mechanisms and host factors) causing an increase in the local pH, that results from a reduction in the hydrogen peroxide-producing lactobacilli. Lactobacilli help maintain the acidic pH of healthy vaginas and inhibit other anaerobic microorganisms. Normally, healthy vaginas have high concentrations of lactobacilli. In BV, the lactobacilli population is reduced greatly, while populations of various anaerobes and *Gardnerella vaginalis* are increased. The anaerobes implicated in BV include *Mobiluncus spp., Prevotella spp., Bacteroides spp., Peptostreptococcus, Fusobacterium*, and *Eubacterium spp. Mycoplasma hominis* and *Ureaplasma urealyticum* have also been implicated. With current culture-independent techniques, a greater number of organisms have been identified as part of the flora found in women with BV, including *Atopobium vaginae* (*2*). Many of the bacteria associated with BV are found in normal women, albeit in smaller numbers; hence, the laboratory diagnosis of BV has been fraught with difficulty, with multiple methods described in the literature. Isolation and identification of individual organisms such as *G. vaginalis* is often proposed but is not appropriate and of no clinical use in the diagnosis of BV (*3*) and could lead to over-treatment. Diagnosis is best achieved either by use of Amsel's clinical criteria (*4*) or by assessment (*5*) or scoring (*6*) of bacteria in a Gram-stained vaginal smear.

> • BV is the most common cause of vaginal discharge and it is related to changes in vaginal ecology, with a reduction in the lactobacilli population of the vagina and an overgrowth of anaerobes and *G. vaginalis*.

7.2 Diagnosis

BV is a clinical entity characterized by increased quantities of malodorous vaginal discharge. The diagnosis is based on the presence of at least three of the following four criteria (Amsel's criteria) (*4*):

- homogeneous white to grey adherent discharge;

- a vaginal fluid pH of >4.5;

- the release of a fishy amine odour from the vaginal fluid when mixed with 10% potassium hydroxide (KOH) solution;

- "clue cells" visible on microscopic examination.

Discharge. The evaluation of this clinical sign is subjective. Discharge in women with BV is often not markedly greater than that seen in healthy women. Moreover, the application of vaginal douches can reduce the amount of discharge.

Vaginal pH. The pH of vaginal fluid should be measured using pH indicator paper strips of appropriate range (3.8 to 6.0) such as Whatman narrow range pH paper. A specimen is collected with a swab from the lateral and posterior fornices of the vagina and the swab is then touched directly on to the paper strip. Alternatively, the pH paper can be touched to the tip of the speculum after it has been withdrawn from the vagina (Fig. 7.1). Contact with cervical mucus must be avoided since it has a pH of >7.0. The normal mature vagina has an acid pH of 4.0. In BV, the pH generally is elevated to >4.5.
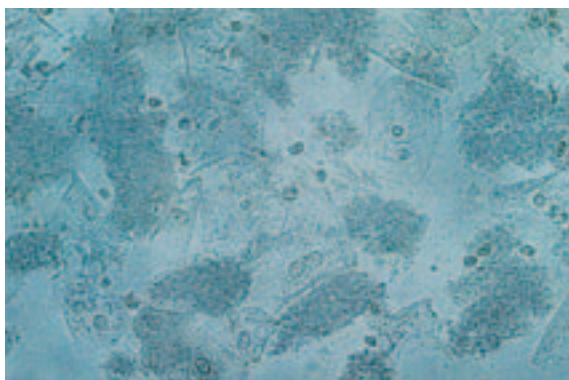

Figure 7.1
pH testing of vaginal fluid, comparing to a standardized colour scale.

The vaginal pH test has the highest sensitivity of the four characteristics, but the lowest specificity; an elevated pH also is observed if the vaginal fluid is contaminated with menstrual blood, cervical mucus, or semen, and in women with a *T. vaginalis* infection.

Odour. Women with BV often complain of a foul vaginal smell. This odour is due to the release of amines, produced by decarboxylation of the amino acids lysine (to cadaverine) and arginine (to putrescine) by anaerobic bacteria. When KOH is added to the vaginal fluid, these amines immediately become volatile, producing the typical fishy odour. Place a drop of vaginal fluid on a glass slide and add a drop of 10% KOH. Hold the slide close to the nose to detect the amine odour. After a positive reaction, a specimen will quickly become odourless upon standing because the amines will be rapidly and completely volatilized. In some parts of the world, KOH is not available due to its caustic nature and so if only three of the four criteria are performed, the sensitivity of the criteria is lost.

Clue cells. Mix a drop of vaginal fluid with a drop of saline on a glass slide. Place a coverslip over the suspension and examine microscopically at 400× magnification. Clue cells are squamous epithelial cells covered with many small coccobacillary organisms, giving a stippled, granular aspect; the edges of these epithelial cells are not clearly defined, owing to the large number of bacteria present and the apparent disintegration of the cells (Figs. 7.2A and 7.2B). In most patients with BV, a mixture of normal exfoliated vaginal epithelial cells and 20% or more clue cells will be seen. The adhering bacteria on the cells are *G. vaginalis* mixed with anaerobes.
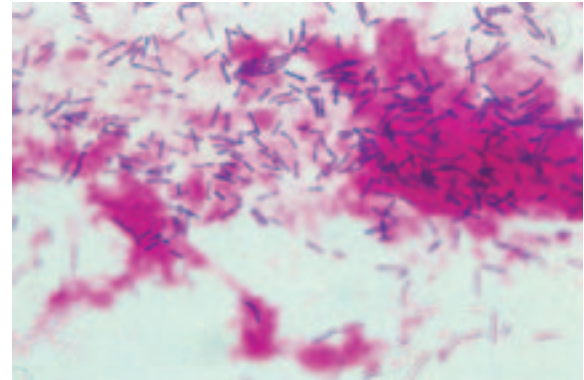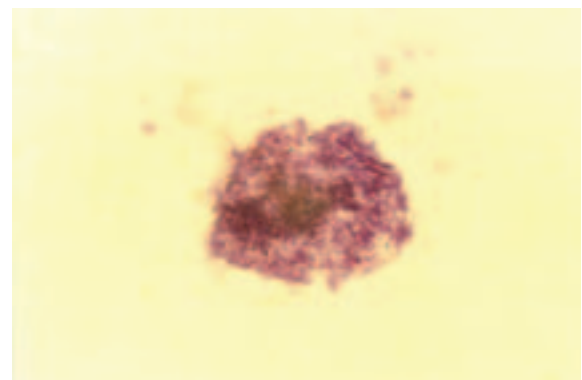

Figure 7.3
Gram stain of normal vaginal smear showing lactobacilli (1000×)
Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

BV is recognizable on a Gram-stained vaginal smear. The slides can be examined in the clinic, where facilities are available, or stored for examination in the laboratory for later independent verification. Grades of vaginal flora ranging from normal (Fig. 7.3) through intermediate BV morphotypes can be seen in the smear. In the smear of a woman with BV, lactobacilli are either absent or reduced in number and are replaced by a mixed microbial flora. Two methods are commonly used for reading smears: Ison–Hay criteria (*5*), which assess the flora and are most suitable for use in routine clinical practice; and Nugent's score (*6*), which scores the individual bacteria and provides a quantitative analysis that is particularly useful for research purposes but offers little advantage for clinical use. Inter-observer variability between the Nugent and Ison–Hay criteria has been reported to be the same (*7*).

A. Clue cells in vaginal wet mount (400×)

B. Clue cell in Gram stain of vaginal smear

Figure 7.2
Microscopy of vaginal smears

7.3 Ison–Hay criteria

This method assesses the relative proportions of the different bacterial morphotypes and grades them as follows:

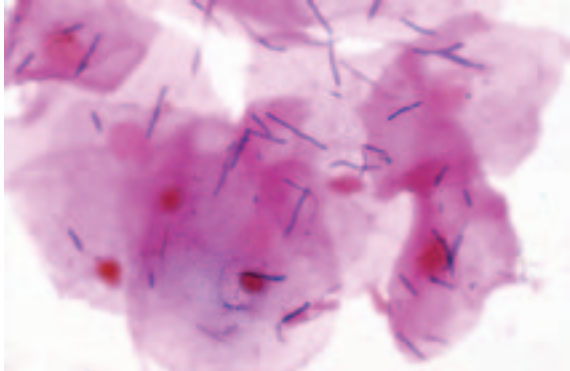Grade I (normal flora), only or predominantly lactobacilli morphotypes (Fig. 7.4)

Figure 7.4
Grade I

Grade II (intermediate flora), reduced lactobacilli morphotypes with mixed bacterial morphotypes (Fig. 7.5)
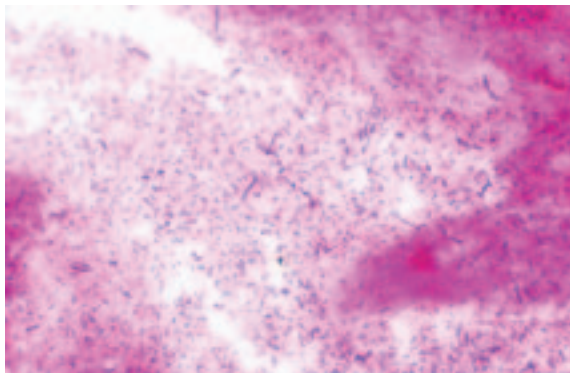

Figure 7.5
Grade II

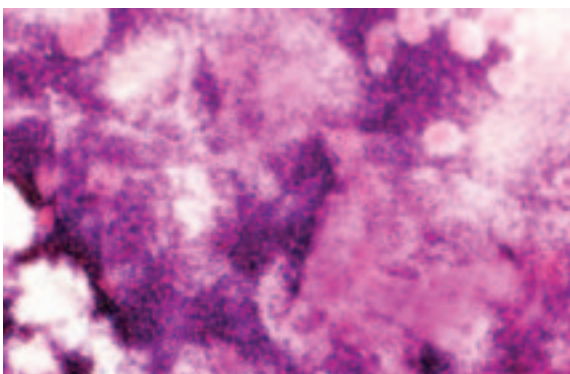Grade III (BV), mixed bacterial morphotypes with few or absent lactobacilli morphotypes (Fig. 7.6)

Figure 7.6
Grade III

Two additional grades in the Ison–Hay system are:
Grade 0, epithelial cells with no bacteria seen (Fig. 7.7)
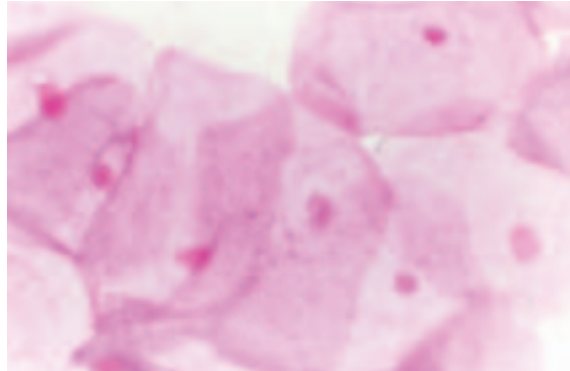
Figure 7.7
Grade 0

Grade IV, epithelial cells covered with Gram-positive cocci only (Fig. 7.8)

Figure 7.8
Grade IV

Both Grade 0 and Grade IV are found in normal women. Grade 0 predominantly follows intravaginal antimicrobial treatment and Grade IV is found in a small number of women who are longitudinally colonized with Gram-positive cocci, usually streptococci, often with no or reduced lactobacilli.

Source (Figs 7.4–7.8): Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

7.4 Nugent's score

This method relies on scoring of individual types of organisms; a score of 0 to 10 is derived from a weighted combination of the follow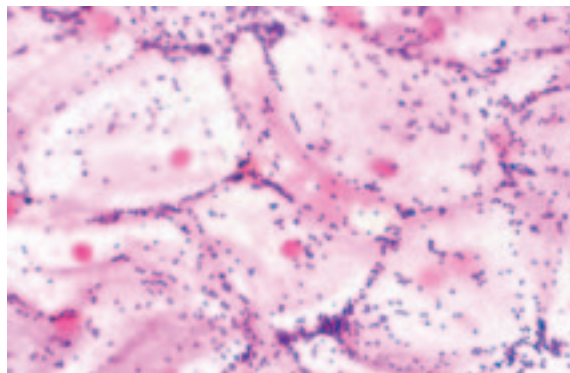ing: large Gram-positive rods (lactobacilli), small Gram-negative or Gram-variable rods (*G. vaginalis* or other anaerobes), and curved Gram-negative or Gram-variable rods (*Mobiluncus spp.*). Each of these three groups is quantitatively weighted on a score of 0–4 on a smear, as follows:

0 = no morphotype per oil field

1+ = less than 1 morphotype per oil field

2+ = 1 to 4 morphotypes per oil field

3+ = 5 to 30 morphotypes per oil field

4+ = more than 30 morphotypes per oil field

Plenty of lactobacilli morphotypes on a smear is considered normal; thus, lactobacilli scores are inversely related to their number. 4+ lactobacillus scores 0, 3+ scores 1, etc. The scores for Gardnerella and Mobiluncus morphotypes correlate to the number of organisms. 4+ Gardnerella scores 4, etc. Mobiluncus are weighted lower; thus, 1+ and 2+ scored organisms score 1, and 3+ and 2+ score 2.

A diagnosis of "severe BV" scores 10 (4 for absence of lactobacilli morphotypes, 4 for 4+ Gardnerella morphotypes, and 2 for 4+ Mobiluncus morphotypes). A "normal" vaginal Gram smear scores 0 (0 for 4+ lactobacilli morphotypes, 0 for 0 Gardnerella morphotypes and 0 for 0 Mobiluncus morphotypes).

In Nugent's score, a total score of 7 to 10 (the sum of the rating scores of the 3 groups described above) is indicative of BV, a score of 4 to 6 intermediate flora, and 0 to 3 normal floras.

7.5 Other tests for BV

Microscopy of a Gram-stained vaginal smear currently remains the preferred laboratory method for the diagnosis of BV. A number of other tests have been described, which may be useful if there is no microscope available. These include:

1. Affirm VP III (*8*)—This uses DNA hybridization to detect high levels of Gardnerella.

2. BV Blue (*9*)—This is a commercially available point-of-care test. It measures sialidase, an enzyme that produces some of the amines released during BV. Compared to the Nugent and Amsel criteria, it has a sensitivity of 88% and specificity of 95% and 91%, respectively.

3. FemExam (*10*)—This consists of two cards with indicators that measure vaginal pH, amines, and enzyme activity. Indicators on FemExam card 1 measure pH greater than or equal to 4.7 and amines with concentration greater than 0.5 mmols. Card 2 measures proline aminopeptidase activity. Compared against Nugent's score, FemExam card 1 has a sensitivity and specificity of 71.4% and 72.8%, respectively, and FemExam 2 sensitivity and specificity of 70% and 81%, respectively.

Molecular methods have recently been used to detect also previously unrecognized bacteria in BV and may have the potential to provide a sensitive and specific diagnosis for BV (*11*, *12*).

- Diagnosis can be made using Amsel's criteria by demonstrating the presence of at least three of the following four criteria: homogenous adherent white to grey vaginal discharge, pH>4.5, amine smell, and clue cells.
- Culture of organisms associated with BV is of no diagnostic value.
- Where facilities are available, a Gram stain of the vaginal discharge showing grades of vaginal flora from normal through intermediate to BV morphotypes can be scored by the Ison–Hay and Nugent criteria for BV diagnosis.

7.6 References

1. Bradshaw CS et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *Journal of Infectious Diseases*, 2006, 193(11):1478–1486.

2. Tabrizi SN et al. Prevalence of *Gardnerella vaginalis* and *Atopobium vaginae* in virginal women. *Sexually Transmitted Diseases*, 2006, 33(11):663–665.

3. Krohn MA, Hillier SL, Eschenbach DA. Comparison of methods for diagnosing bacterial vaginosis among

pregnant women. *Journal of Clinical Microbiology*, 1989, 27(6):1266–1271.

4. Amsel R et al. Non specific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *American Journal of Medicine*, 1983, 74(1):14–22.

5. Ison CA, Hay PE. Validation of a simplified grading of Gram stained vaginal smears for use in genitourinary medicine clinics. *Sexually Transmitted Infections*, 2002, 78(6):413–415.

6. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *Journal of Clinical Microbiology*, 1991, 29(2):297–301.

7. Forsum U et al. An international study of the interobserver variation between interpretations of vaginal smear criteria of bacterial vaginosis. *APMIS*, 2002, 110(11):811–818.

8. Gazi H et al. Use of DNA hybridization test for diagnosing bacterial vaginosis in women with

symptoms suggestive of infection. *APMIS*, 2006, 114(11):784–787.

9. Bradshaw CS et al. Evaluation of a point-of-care test, BVBlue, and clinical and laboratory criteria for diagnosis of bacterial vaginosis. *Journal of Clinical Microbiology*, 2005, 43(3):1304–1308.

10. West B et al. Evaluation of a new rapid diagnostic kit (FemExam) for bacterial vaginosis in patients with vaginal discharge syndrome in the Gambia. *Sexually Transmitted Diseases*, 2003, 30(6):483–489.

11. Fredricks DN et al. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *Journal of Clinical Microbiology*, 2007, 45(10):3270–3276.

12. Shipitsyna E et al. Composition of the vaginal microbiota in women of reproductive age—sensitive and specific genetic diagnosis of bacterial vaginosis is possible? *PLoS ONE*, 2013, 8(4):e60670.

Chapter 8

Candidiasis

8.1 Introduction

Vulvovaginal candidiasis (VVC) is caused by the fungus *Candida albicans* (*1*) in approximately 85% of cases, with *C. glabrata* being responsible for the remaining 15% (*2*). Other species, such as *C. krusei*, and *C. tropicalis* rarely cause vaginitis (*3*). *Candida spp*. are usually of endogenous origin and can be isolated from the genital tract in up to 25% of asymptomatic healthy women of childbearing age. For *Candida spp*. to colonize the vagina, they must first adhere to the vaginal epithelial cells and then grow, proliferate, and germinate, before finally causing symptomatic inflammation. Changes in the vaginal environment are usually necessary before the organism can induce pathological effects. Probably the most important defence against both colonization and inflammation is the natural bacterial flora. The mechanism whereby Candida induces inflammation has not yet been established, but important predisposing factors for colonization and inflammation include:

- changes in reproductive hormone levels associated with premenstrual periods, pregnancy, and oral contraceptives;

- the use of antibiotics;

- Diabetes mellitus;

- immunosuppression.

Candidiasis is usually of endogenous origin and not sexually transmitted. Chronic or recurrent VVC occurs in a small number of women, causing persistent symptoms, and can be associated with the risk factors described above (*4*, *5*). Chemical products, local allergy and delayed hypersensitivity may also contribute to the induction of symptomatic vaginitis and vulvitis.

In men, the significance of *Candida spp*. is unclear, although it may be transmitted between sexual partners and can cause balanitis or balanoposthitis and rarely urethritis. Typically, men develop an allergic response to candidal antigen although fulminant infection may be seen more frequently in patients with the aforementioned risk factors (*6*).

8.2 Diagnosis

The diagnosis of VVC is usually established on the combination of clinical manifestations and microscopy of a wet mount preparation. Classic symptoms and signs of VVC include vaginal itching, an odourless curdy white discharge ("cottage cheese"), a burning sensation in the vulva, dysuria, and erythema of the labia and vulva. Symptoms and signs, however, are often more equivocal. Detection of budding yeast cells by wet mount or potassium hydroxide (KOH) microscopy can be performed in the laboratory or the clinic and has a very high predictive value for the diagnosis of VVC. While this combination is generally used, in women with classic signs it is often reasonable to give therapy on a presumptive clinical diagnosis without further confirmation by microscopy. Use of a Gram-stained smear and detection of budding yeast cells and pseudohyphae is preferred in some centres for the determination of candidiasis.

In women with abnormal vaginal discharge, and in the absence of a microscope, the detection of a pH of <4.5 is a good indicator of VVC and can help to differentiate it from bacterial vaginosis and trichomoniasis, both of which typically produce a pH of >4.5. A narrow range pH paper (Whatman) is an inexpensive, sensitive, and simple method to use and is available in most settings.

Culture is the most sensitive method currently available for the detection of *Candida spp.*, but it must be used with caution as *Candida spp.* are also found in women without VVC. Therefore, culture should be considered only if VVC is clinically suspected but microscopy is negative (although in this instance presumptive treatment may be appropriate and uses less money and fewer resources), or when antimicrobial susceptibility testing is required.

Molecular detection of *Candida spp.* using polymerase chain reaction (PCR) has been described but offers no advantage over currently available tests, as the high sensitivity demonstrated by PCR will detect yeasts of *Candida spp.* in women without VVC and will result in over-diagnosis and unnecessary treatment.

8.3 Collection of specimens

Obtain a sample of discharge from the lateral vaginal wall with a swab—the type of fibre is not important. In patients who have only a slight vaginal discharge and extensive involvement of the vulva or labia, it is better to collect a specimen from the irritated mucosa. Direct microscopy can be done immediately at the clinic site or the specimen may be transported to the laboratory. The use of a transport medium, such as Amies, is not necessary if yeasts have to be identified, but is to be preferred to maintain viability and motility of trichomonads.

In males with balanitis, use a swab pre-moistened in saline to collect the sample from the glans penis.

8.4 Direct microscopy

Place the specimen on a glass slide and, if necessary, depending on its fluidity, mix with a drop of saline. Cover the preparation with a cover slip and examine microscopically at 400× magnification not only to detect yeast cells, but also to assess the presence of trichomonads and clue cells. Yeasts are round to ovoid cells, 4 µm in diameter, showing typical budding (blastoconidia) (Figs. 8.1A and 8.1B). The addition of 10% KOH to the preparation increases slightly the detection sensitivity of yeasts, making the recognition of mycelia (pseudohyphae) much easier. Yeasts can easily be recognized on a Gram-stained smear as they are Gram-positive cells (Figs. 8.2A and 8.2B).
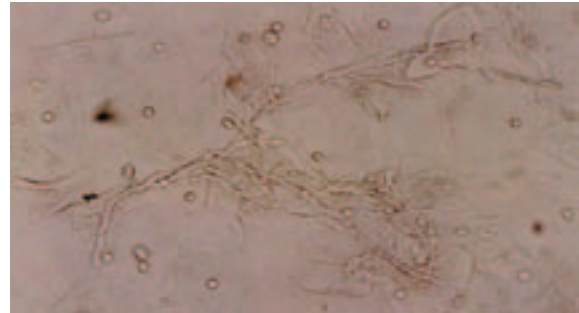
Figure 8.1A
Potassium hydroxide (KOH) preparation of vaginal fluid showing budding yeasts and pseudohyphae (400×)
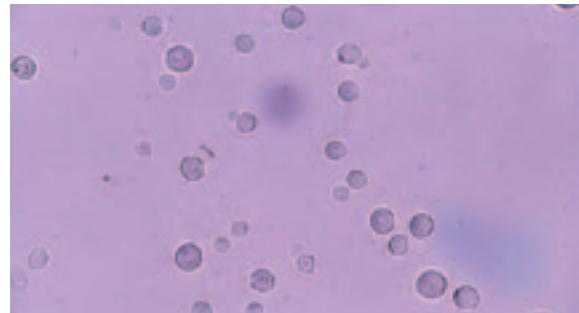
Figure 8.1B
Wet mounts of yeasts without pseudohyphae
Source (A and B): Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

Figure 8.2A
Gram stained smear of vaginal discharge showing Gram-positive yeasts and pseudohyphae (1000×)

Figure 8.2B
Budding yeasts without pseudohyphae
Source (A and B): Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

8.5 Culture

Sabouraud dextrose agar with chloramphenicol is an excellent growth medium for the isolation of *Candida spp*. After inoculation of the clinical specimen, the plates are incubated at 36°C for 2 days. Colonies of yeast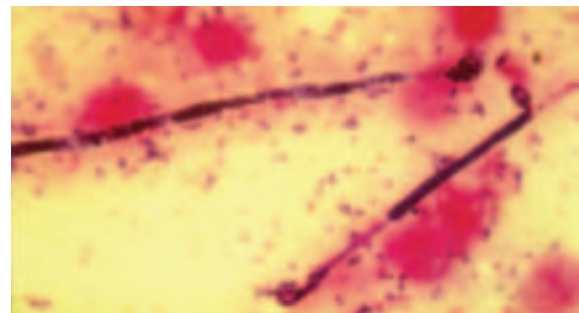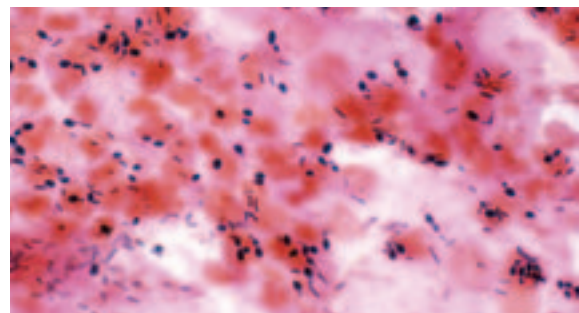 cells are opaque and white to creamy in colour. The only important identification for routine diagnostic purposes is a differentiation from bacteria. Microscopy can be used to confirm the presence of yeast cells.

Further identification of yeasts is not necessary for routine diagnosis of uncomplicated VVC. However, the germ tube test is a simple test for the presumptive identification of *C. albicans*. A colony is emulsified in 0.5 ml of bovine or horse serum and incubated at 36°C for 4 hours. *C. albicans* will show short lateral hyphal filaments without any constrictions. A complete identification of yeasts to species level can be obtained by means of auxanographic methods for carbohydrate and nitrate assimilation, or through carbohydrate fermentation tests. Kits for the identification of yeasts to species level are commercially available.

- The diagnosis of candidiasis is established with a combination of clinical features and microscopy of a sample of an appropriate specimen.
- Culture of the genital discharge should be considered for a negative microscopy in the presence of clinical symptoms.
- Antimicrobial susceptibility testing of *Candida spp.* is occasionally warranted and should be performed in specialist centres.

8.6 References

1. Sobel JD. Vulvovaginal candidosis. *The Lancet*, 2007, 369(9577):1961–1971.

2. Sobel JD. Vulvovaginitis due to *Candida glabrata*. An emerging problem. *Mycoses*, 1998, 41(Suppl 2):18–22.

3. Horowitz BJ, Giaquinta D, Ito S. Evolving pathogens in vulvovaginal candidiasis: implications for patient care. *Journal of Clinical Pharmacology*, 1992, 32(3):248–255.

4. Morton RS, Rashid S. Candidal vaginitis: natural history, predisposing factors and prevention. *Proceedings of the Royal Society of Medicine*, 1997, 70(Suppl 4):3–6.

5. Sobel JD. Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clinical Infectious Diseases*, 1992, 14(Suppl 1):S148–S153.

6. Marrazzo J, Hillier S, Sobel J. Vaginal infections. In: Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/ Elsevier, 2010:76–93.

Chapter 9

Herpes simplex virus (HSV) infections

9.1 Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are large double-stranded DNA viruses. HSV-1 and HSV-2 share a similar genome structure, with 40% of sequence homologies and 83% homology of their protein-coding regions, which explains numerous biological similarities and antigenic cross-reactivity between the two serotypes.

During primary infection, HSV enters through breaks in the skin or mucosa; it then attaches to and enters epithelial cells and begins replicating. It is then taken up by free sensory nerve endings and transported to the sensory ganglion serving that area of skin. Skin manifestations include vesicular lesions leading to shallow ulcerations that crust and heal spontaneously within 2 to 3 weeks without scarring. Lesions lead to the focal destruction of the epithelial layer and an infiltration of inflammatory cells develops in the surrounding rim and in the underlying dermal layer. However, only 10–30% of new infections are symptomatic (Fig. 9.1).

After recovery from the initial infection, the virus remains latent in the sensory ganglion for the life of the host. Periodically, the virus may reactivate from the latent state and travel back down the sensory nerves to the skin or mucosal surface. Intermittent viral shedding can occur either in the presence of lesions (clinical reactivation) or with very mild or no symptoms (subclinical reactivation). Shedding from mucosal surfaces leads to transmission to sexual partners.

Symptomatic recurrent episodes tend to be milder that primary episodes, normally healing within 10 days, but can be severe, particularly in immunocompromised individuals. Table 9.1 summarizes the main clinical symptoms, manifestations, and complications of genital herpes infections. Approximately 70–90% of people with symptomatic genital HSV-2 and 20–50% with symptomatic genital HSV-1 will have a recurrence within the first year. Immunocompetent people with genital herpes can have frequent, painful, and recurrent genital lesions associated with much psychosocial distress
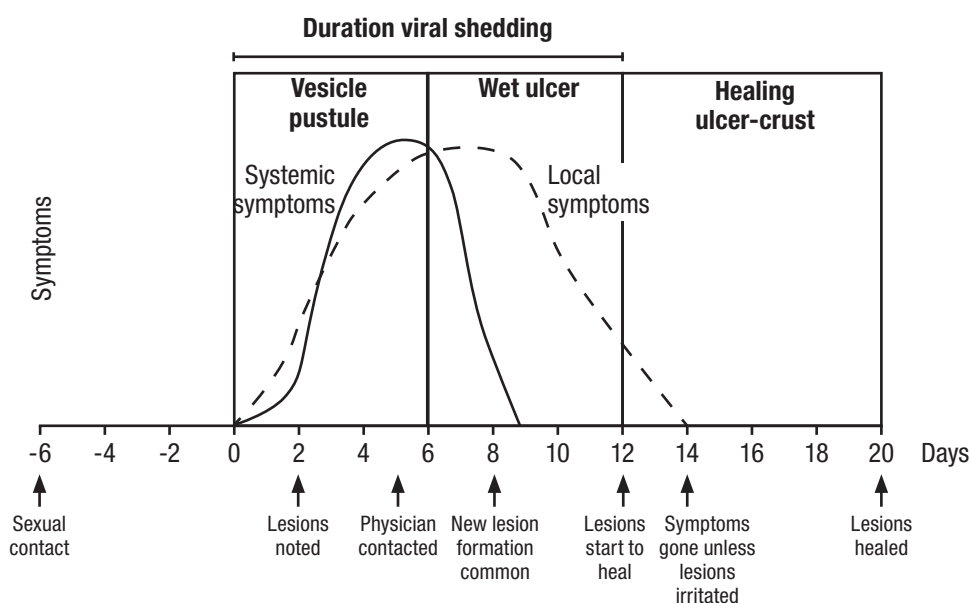

Figure 9.1
Clinical course of primary genital herpes

The classical pattern of HSV-1 and HSV-2 infections associated with oral or genital diseases, respectively, remains the rule in certain parts of the world, such as sub-Saharan Africa where HSV-1 infection remains a common disease of childhood and HSV-2 infection is sexually transmitted. In contrast, the differentiation of HSV-1 from HSV-2 based on anatomical site of infection is far from absolute in developed countries, since genital herpes frequently may be caused by HSV-1. The delay in acquisition of oral HSV-1 infection early in life in developed countries renders a significant proportion of young adults susceptible to genital HSV-1 infection following initiation of sexual activity and oro-genital sexual contact, which is viewed as safer than penetrative sexual intercourse and a means of averting pregnancy. In developing societies, rates of HSV-1 seropositivity often reach 100% among adults but only 50–70% in developed countries, where an upsurge in genital HSV-1 infections has been reported in the past decade.

Genital HSV-1 infections now account for at least half of all first episode genital herpes in young adults in western societies. In addition, HSV-2 seropositivity increases after sexual debut and steadily increases with age. HSV-2 seroprevalence varies by regions of the world and ranges from approximately 10–40% in adults, and may reach 60–95% in HIV-infected individuals and female sex workers. HSV-2 shares risk factors with the other sexually transmitted infections (STIs), namely, high number of lifetime sexual partners, previous history of STIs, and early sexual debut. The incubation period of both HSV-1 and HSV-2 is usually from 2 to 10 days (up to 4 weeks). In both men and women with primary genital herpes, lesions start as papules or vesicles to give rise to pustular and ulcerative lesions. These ulcerative lesions persist for 4–15 days until they crust and finally re-epithelialize.

Table 9.1: Clinical symptoms, signs, and complications during symptomatic genital herpes infections

	Clinical symptoms	Clinical signs	Complications
Males	• Papular or vesicular lesions on genitals, perigenital areas, or extragenital areas (thigh, eye, buttock, finger) • Pustular lesions • Genital ulceration • Perineal pain • Dysuria • Inguinal discomfort or pain (peri-adenitis) • Pharyngeal infection (oro-genital contact)	• Papular, vesicular, pustular lesions followed by ulcerations on penis, perineum, thigh • Urethral discharge • Urethritis	• Aseptic meningitis • Extensive vesicular skin rash • Urinary retention • Sensitive radiculopathy by involvement of sacral nerves • Transverse myelitis • Increased risk for acquiring HIV by sexual exposure • Increased risk of sexual transmission of HIV in HIV/HSV-coinfected individuals • Neonatal herpes after vaginal delivery
Females	• Papular or vesicular lesions on genitals or perigenital areas or extragenital areas (thigh, eye, buttock, finger) • Pustular lesions • Genital ulceration • Vaginal or cervical discharge • Perineal pain • Dysuria • Inguinal discomfort or pain (peri-adenitis) • Dyspareunia • Pharyngeal infection (oro-genital contact) • Primary infection is often worse in intensity and duration in women than in men	• Papular, vesicular, pustular lesions followed by ulcerations on vulva, perineum, thighs • Vaginal or cervical discharge • Cervicitis • Hyperemia of the mucous membranes of vulva and vagina • Urethral discharge • Urethritis	

HIV, human immunodeficiency virus; HSV, herpes simplex virus.

The most serious consequence of genital herpetic infection is the possibility of vertical transmission from an infected mother to her neonate during vaginal delivery. This can result in disseminated infection, central nervous system involvement, and possibly neonatal death. In addition, throughout the past two decades, HSV-2 infection also has been linked to a threefold greater risk of HIV acquisition. Mucosal disruption caused by the genital ulcers favours HIV acquisition by providing a ready portal of entry. Moreover, HSV-2 reactivation results in mucosal infiltration with activated CD4-bearing lymphocytes, the target cells for HIV-1 attachment. These same factors facilitate spread of HIV infection from an HSV/HIV-coinfected individual to an uninfected sexual partner.

9.2 Overview of diagnostic procedures

Genital herpetic infection often is diagnosed on clinical grounds as a result of the presence of a cluster of vesicular lesions. However, since other causes of genital ulceration may have a similar clinical presentation, particularly if only ulcerations are noticed, laboratory confirmation of the diagnosis may be necessary. Laboratory methods used for the diagnosis of HSV infection include direct detection of HSV in material from lesions and indirect serological methods (Tables 9.2 and 9.3). Available tests for HSV include antigen detection, viral culture, and nucleic acid amplification tests (NAAT) for viral DNA, as well as the use of serological assays to screen for exposure to HSV by detecting HSV-type-specific antibodies. Viral culture with further herpes typing has been the cornerstone of HSV diagnosis throughout the past two decades. However, detection of HSV DNA in clinical specimens using amplified molecular testing has now emerged as an alternative method because it is up to four times more sensitive, less dependent on collection and transportation conditions, and more rapid than viral culture. The choice of test and how to interpret the results are important considerations for both microbiologists and their clinical colleagues.

9.3 Specimen collection and transportation

HSV-1 and HSV-2 can be recovered by swabbing mucocutaneous genital lesions and from previously involved mucocutaneous sites in patients with asymptomatic infection. For sample collection, a small cotton-tipped or Dacron swab on a wire shaft is used for viral culture as well as molecular biology. Calcium alginate swabs reduce viral recovery and inhibit NAAT-based detection methods. Because HSV genital shedding is intermittent, testing swabs from asymptomatic patients is not recommended for routine diagnosis since this approach is unlikely to provide confirmation of carrier status.

For recent lesions, collection of vesicular fluid or exudate from small vesicles is the method of choice. Vesicular fluid from large vesicles containing a high concentration of virus may be aspirated with a tuberculin syringe. After aspiration, the vesicle surface is removed and the base of the lesion is swabbed vigorously to recover infected epithelial cells. For older lesions, the diagnostic yield may be unacceptably low. However, individuals with such lesions should be advised to return when fresh new lesions are present for sampling. Occasionally, other anatomical sites may be sampled with a swab, for example, the urethra, vagina, or cervix.

Careful attention should be given to the conditions of transport and storage of clinical specimens. After sampling, specimens for viral detection should be placed immediately into vials containing 1 ml of appropriate viral transport medium for cell culture, a universal transport medium (for culture or NAAT), or sent as a dry swab (for NAAT only). Culture is recommended in situations where it is possible to send clinical specimens to the laboratory within 4 hours. Failing this, specimens may be placed at 4ºC overnight and sent the next day. The transport time from specimen collection to laboratory should be no more than 48 hours and, in such situations, it is essential to send the samples to the laboratory on ice in a cooler box. It is important to note that virus recovery is substantially reduced following a freeze/thaw cycle and that freezing at −20°C pending transport to the laboratory is not advised. The recommended sampling sites and type of sample and methods to be used for the diagnosis of genital herpes infection are presented in Tables 9.2–9.4. Table 9.3 presents the recommendations for sample transportation and storage using microscopy, culture, and NAAT.

Table 9.2: Recommendations for sample collection for the diagnosis of genital herpes infections, adapted from Domeika and colleagues (*1*)

Collection site	Tools for sample collection	Collection method

Male skin or mucous membrane lesions (including the perianal region)	• Sterile needles
	• Sterile cotton-tipped, Dacron, or nylon-flocked swab on a wooden, plastic, or aluminium shaft
	• Microscope slides
		• Unroof the vesicles with a sterile needle
		• Collect the content of the vesicles with a sterile swab and:
		  – apply to a microscope slide (for immunofluorescence staining) OR
		  – introduce into transport media for viral culture or NAAT

Male urethra	• Sterile cotton-wool, Dacron, or nylon-flocked swab on a wooden, plastic or aluminium shaft	• Clean the external urethral opening region with a swab moistened in saline • Draw back the prepuce to avoid contamination when sampling • Insert a sterile swab carefully into the external urethral meatus (to a depth of 0.5–2 cm) and collect urethral exudates for testing
Female skin or mucous membrane lesions (including the perianal region)	• Gauze and cotton swabs • Microscope slides	• Similarly as for male skin or mucous membrane lesions
Female urethra	• Sterile gauze swab (to remove excess discharge) • Sterile cotton-wool, Dacron, or nylon-flocked swab on an aluminium shaft	• Clean the introitus using a sterile gauze swab • Carefully insert a sterile swab on an aluminium shaft into the urethra (to a depth of 0.5 cm) to collect exudates for testing
Cervix	• Vaginal speculum • Sterile gauze swab • Sterile cotton-wool, Dacron, or nylon-flocked swab on a wooden or plastic shaft	• Insert the vaginal speculum, which may be moistened in advance with warm water, and clean the cervical canal opening thoroughly with a sterile gauze swab • Insert a cotton-wool or Dacron swab carefully into the cervical canal (to a depth of 2 cm) and collect the material from lesions

NAAT, nucleic acid amplification test.

Note: Material from the rectum is collected when the patient has had anal sexual contact, when there is anorectal inflammation, or if perianal skin or anal folds are thickened.

Table 9.3: Direct laboratory methods for HSV diagnosis

Method	Principle	Sample	Sensitivity	Specificity	Advantages	Disadvantages
Cytological examination	Tzanck smears, Papanicolaou (Pap), or Romanovsky stain	• Skin/ mucosal lesions • Biopsies • Conjunctival/ corneal smears	Low	Low	• Inexpensive	• Fresh lesions • Suboptimal sensitivity and specificity
	Detection of infected cells by direct immuno-fluorescence	• Smears, tissue section • Smear from base of vesicle	Middle (genital ulcer: 70–90%; asymptomatic: <40–50%)	High (>95%)	• Inexpensive • Rapid (<4 hours possible) • Typing possible	• Fresh vesicles • Suboptimal sensitivity • Time-consuming • Labour-intensive • Not standardized
Viral antigen detection	Immuno-peroxidase staining	• Swab • Smears from lesions • Smear or vesicular fluid of exudate from base of vesicle	Middle (80%)	High (90%)	• Reagent cost • Rapid (<4 hours possible) • Does not require the integrity of the specimen • Typing possible	• Fresh vesicles • Suboptimal sensitivity
	Capture ELISA	• Swab • Vesicular fluid or exudate from base of vesicle	High (genital ulcer: >95%)	High (62–100%)		• Fresh vesicles • No viral typing
	Rapid test device	• Swab • Vesicular fluid or exudate from base of vesicle	Unknown	Unknown	• Point-of-care testing	• Not yet evaluated

Table 9.3: Direct laboratory methods for HSV diagnosis (*continued*)

Method	Principle	Sample	Sensitivity	Specificity	Advantages	Disadvantages
Virus culture	HSV isolation susceptible cells	• Swab • Skin lesions • Vesicular fluid or exudate from base of vesicle • Mucosal sample without lesions • Biopsies • Conjunctival/ corneal smear • Neonates	• Low to high depending on the clinical context • Vesicular content: >90% • Ulcer: 95% • Swab: 70–80% • Mucosa without lesion: 30%	High (~100%)	• Allows virus • Classically, "gold standard" method • Currently, "preferred" test (*2*) • Simplicity of sampling • Virus typing • Resistance phenotype testing[a]	• Less sensitive than PCR • Sample storage and transport conditions influence sensitivity (rapid transport, cooled, protected from light in virus transport medium) • Labour-intensive • Expensive • Specialized laboratories • Results in 2–7 days • Arrangement with laboratory necessary

Table 9.3: Direct laboratory methods for HSV diagnosis (*continued*)

Method	Principle	Sample	Sensitivity	Specificity	Advantages	Disadvantages

Molecular biology	HSV DNA detection and/or quantitation by NAAT, including in-house classical PCR, real-time PCR and commercial assays	• Swab
		• Skin lesions
		• Vesicular fluid or exudate from base of vesicle
		• Mucosal sample without lesions
		• Aqueous/ vitreous humor
		• Cortico-spinal fluid
		• Blood
			Highest (98%)	High (~100%) Containment of potential cross-contamination important	• High sensitivity
					• Currently, "preferred" test (*2*)
					• Allows virus detection and typing in the same test
					• Rapid
					• May be automated
					• Labour-efficient
					• Result within 24–48 h, possibly in <3 h
					• Resistance genotyping
					• Method of choice for CSF
					• Real-time PCR:
					  – Rapid amplification
					  – Quantitative analysis
					  – Reduced risk of contamination
					  – Method of choice for skin lesions
						• Only in specialized laboratories
						• Not standardized
						• Not validated for all samples
						• Risk of contamination (PCR)
						• May be relatively expensive (real-time PCR)
						• Routine resistance genotyping not available

CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; HSV, herpes simplex virus; NAAT, nucleic acid amplification test; PCR, polymerase chain reaction.

[a] The detection of resistance mutations encoding resistance to anti-herpetic drugs (acyclovir) by HSV drug resistance genotyping is likely to supplant phenotypic testing in the next few years.

9.4 Direct diagnosis of HSV from clinical specimens

9.4.1 Cytological examination

Direct examination of specimens and cytological examination using conventional staining procedures (Tzanck smears, Papanicolaou, or Romanovsky stains) have been found to have low sensitivity and specificity and should not be relied upon for diagnosis of herpesvirus infection. However, a diagnosis of herpes cervicitis may be an incidental finding during examination of routine Pap smears submitted for cervical cytology.

9.4.2 Viral antigen detection

When mucocutaneous lesions are present, viral antigen in lesional material can be detected using direct immunofluorescence (IF), immunoperoxidase (IP) staining, or enzyme-linked immunosorbent assay (ELISA).

Direct IF could be classified as a rapid diagnostic test, allowing type differentiation of genital herpesviruses using clinic-prepared smears or laboratory-prepared smears from swabs transported to the laboratory. For the latter, cells should be concentrated before smears are prepared. HSV-1 and HSV-2 antigens may be detected by type-specific fluorescein-labelled monoclonal antibodies (Fig. 9.2). The main limitations of direct IF are that it is time-consuming, relatively expensive in terms of reagents and capital equipment (a fluorescence microscope), and less sensitive than modern molecular methods. Direct IF has a sensitivity of 70–90% when compared to culture in cases of symptomatic disease; the sensitivity is much lower in asymptomatic cases, precluding its use in such situations.

Indirect IP staining has the advantage of using a normal light microscope, making it more suitable for intermediate-level laboratories. The specimens are prepared as for the direct IF test and incubated with rabbit or mouse HSV-specific polyclonal or HSV-1-or HSV-2-specific monoclonal antibodies. Binding is detected using horseradish-peroxidase-labelled antibody directed to the rabbit or mouse immunoglobulins using standard IP methods. The sensitivity of direct IF and IP methods are similar.

Herpetic proteins also can be detected in clinical samples by using a classical capture ELISA employing HSV-specific polyclonal or monoclonal antibody. The sensitivity of commercially available capture ELISA, as compared with that of viral culture, is greater than or equal to 95% with specificities ranging from 62% to 100% in symptomatic patients. The sensitivity of antigen capture ELISA may be higher than that of virus culture for typical presentations, but lower for cervical and urethral swabs. Most commercially available assays, however, do not differentiate between HSV-1 and HSV-2.

Rapid point-of-care (POC) tests for HSV antigen detection are commercially available, but their performance has not been widely evaluated.

9.4.3 Virus isolation and typing in cell culture

The most frequently used cells for the isolation of HSV from clinical specimens include primary human diploid fibroblasts and cell lines, such as MRC-5 cells (human fibroblasts), Vero cells (monkey kidney), Hep-2 cells (laryngeal squamous cell carcinoma), baby hamster kidney, and rabbit kidney cells.

Cultured cells are allowed to grow into a confluent monolayer in a tissue culture tube flattened on one side. The culture growth medium is removed, and two culture tubes are inoculated with 0.25 ml aliquots of vortexed specimens. The tubes are placed in a horizontal position in an incubator for 1 hour at 36°C to enhance absorption of the viral particles by the cells. After absorption, 2 ml of herpes cell maintenance medium are added, and the culture tubes are incubated at 36°C in an atmosphere containing 5% CO_2 for a period of 7 days. The culture tubes should be examined daily using a stereoscopic microscope to check for the appearance of the
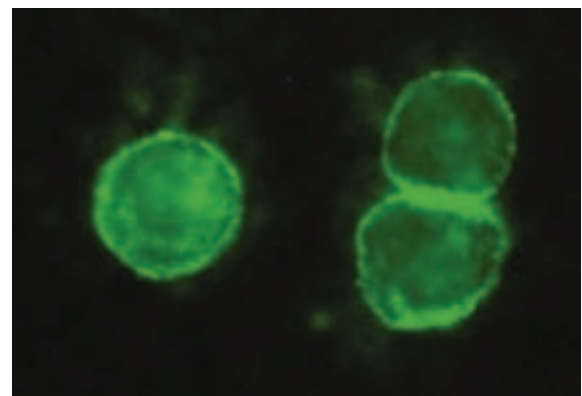

Figure 9.2
Intracellular immunofluorescence of HSV in a clinical specimen (400×)

characteristic cytopathic effect of HSV, which usually develops 24–72 hours after inoculation. The cytopathic effect caused by HSV is characterized by the transformation from elongated, scattered cells (Fig. 9.3, left) to enlarged, refractile, rounded cells, ballooning, increasing in number, and developing a granular appearance (Fig. 9.3, right). Focal necrosis of cells may occur and syncytia and multinucleated giant cells may be found at the edge of the foci in HSV-infected cell cultures.

Confirmation of HSV in cell cultures demonstrating a cytopathic effect is recommended since other viruses may cause a cytopathic effect similar to that observed in herpes cultures. Confirmation and HSV typing can be performed directly on infected cell cultures using fluorescein isothiocyanate (FITC)- or IP-labelled type-specific monoclonal antibodies directly on a smear of cultured material or by testing the cell supernatant by a NAAT. Identification and typing by direct IF is the most practical approach. The culture medium is removed from the infected monolayer and 1 ml of 5% fetal calf serum in phosphate-buffered saline (PBS) solution is added. The cells of the flat monolayer side are then scraped off the coverslip and homogenized and centrifuged at 500g for 5 minutes and 1 drop of the vortexed pellet spotted onto each of 2 wells of a PTFE-coated glass slide, dried in air, and fixed with cold (2–8°C) acetone for 10 minutes. One well is then stained with fluorescein-labelled HSV-1-specific monoclonal antibody, and the other with fluorescein-labelled HSV-2-specific

monoclonal antibody. The slide is incubated for 30 minutes at ambient temperature in a moist chamber, and then washed 3 times for 5 minutes with PBS, using a mechanical stirrer. Coverslips subsequently are placed on the slides using a glycerol–PBS (50%:50%) solution, and the slides examined under a fluorescence microscope at 400× magnification. When one of the spots shows apple-green fluorescent particles while the second does not, the identification of HSV is confirmed and the virus type determined. Isolates of cultured HSV may be stored in 0.2 M sucrose in 0.02 M PBS pH 7.2 (2SP medium) at −80°C or in liquid nitrogen.

Virus isolation in tissue culture roller tubes is slow and labour–intensive, but has the advantage of demonstrating active infection within a clinical lesion and also allows virus typing and antiviral phenotypic sensitivity testing. More rapid culture of HSV can be achieved by using shell vials or multiwell plates, and centrifuging the specimen onto cell monolayers on coverslips in a similar manner to chlamydia culture.

Diagnosis of HSV infection with tissue culture has low sensitivity because HSV is isolated from lesions in approximately 80% of primary infections but in only 25–50% of recurrent lesions, and in even fewer people whose lesions have begun to heal.

Thus, fluid collected from intact blisters (vesicular or pustular lesions) will yield culture isolates in more than 90% of cases. By the time the lesions have crusted, only approximately 25% of cultures will be positive. Failure to
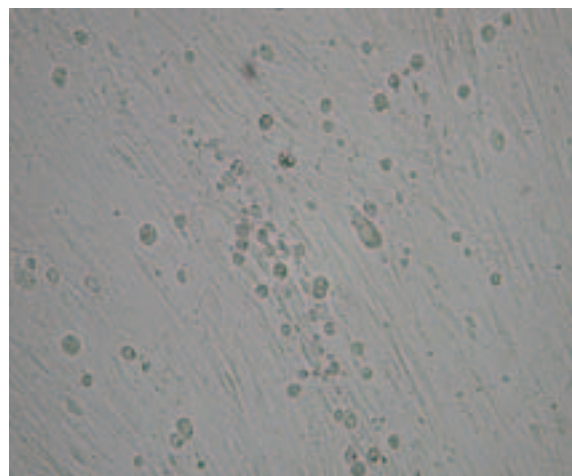

Figure 9.3
Herpesvirus isolation on cell culture (50×). Left, confluent monolayer of uninfected MRC-5 human diploid fibroblasts; right, typical cytopathic effect of HSV on MRC-5 cells obtained after 24–48 hours of viral culture.

detect HSV by culture does not indicate an absence of HSV infection.

HSV isolation in cell culture has been the cornerstone of HSV diagnosis but is feasible only in specialized laboratories. The advantages of HSV culture include high specificity and recovery of viral isolates that can be typed and tested for phenotypic antiviral susceptibility. HSV culture is expensive, labour-intensive, time-consuming, and has a lower diagnostic sensitivity than NAAT assays. Delayed sample processing and lack of cold chain after collection significantly reduce diagnostic yield.

9.5 Virus detection and quantification using molecular techniques

Several molecular procedures have been proposed to detect and/or quantify HSV genomes in clinical samples, including in-house competitive polymerase chain reactions (PCRs) (*3*), PCR detection followed by DNA enzyme immunoassay hybridization (*4*), real-time PCR assay (*5–8*), and various commercially available kits. Real-time NAATs allow both the detection and the quantification of HSV DNA in clinical samples. Compared with traditional NAATs, real-time PCR allows HSV DNA amplification in a single reaction tube, is faster, allows simplified conditions of performance, and lowers the risk of cross-contamination. Primers from HSV DNA sequences common to both HSV-1 and HSV-2 (HSV DNA polymerase [*pol F* gene], HSV thymidine kinase, or glycoprotein B domain) may identify HSV DNA (*6–8*). Primers and probes for HSV DNA sequences specific for HSV-1 or HSV-2, including gG, gD, or gI genes, permit amplification of one herpes type. In each DNA extraction and subsequent analysis, an internal positive control, which permits the detection of amplification inhibitors samples and controls the quality of sample preparation, and a negative control are necessary. Certified and registered reference panels comprising coded control specimens ideally should be used as intra- and inter-laboratory quality controls (see Chapter 2).

NAATs are the most sensitive test currently available to detect HSV in genital samples. The detection rates of the PCR assays were shown to be 11–71% superior to virus culture. Furthermore, NAAT allows detection of asymptomatic HSV shedding. However, failure to detect HSV by PCR does not indicate an absence of HSV

infection because viral shedding is intermittent. The use of NAATs for HSV diagnosis also allows less stringent sample transportation conditions than those required for diagnosis by culture. Real-time PCRs offer the additional advantages of rapid detection, quantification, and typing of HSV DNA without risk of contamination in one-tube procedures.

Although the Food and Drug Administration has not yet approved any NAAT for use in the United States of America, the 2010 Centers for Disease Control and Prevention Sexually Transmitted Diseases Treatment Guidelines state that "PCR testing to diagnose herpes can be performed by clinical laboratories that have developed their own tests and have conducted a clinical laboratory improvement amendment verification study", and "cell culture and PCR are the preferred HSV tests for persons who seek medical treatment for genital ulcers or other mucocutaneous lesions" (*2*). Strict validation of in-house PCRs or commercially available NAATs in individual settings is important.

9.6 Indirect serological diagnosis of herpetic infections

Serological testing is recommended as an aid to diagnosis of genital herpes in patients with recurrent genital symptoms, atypical lesions, or with healing lesions and negative HSV cultures. In the absence of lesions or with negative virus detection tests, serological testing can be useful for the management of sex partners of people with genital herpes and for identifying HSV infection in at-risk individuals such as patients with HIV or other STIs and individuals who have had multiple sexual partners. Serological screening for HSV-1 or HSV-2 in the general population is not indicated.

Although the accuracy of HSV serological assays might not match that of HIV antibody tests, these tests are clearly an improvement on clinical diagnosis of genital herpes, which has a sensitivity of 39% at best and yields a false-positive diagnosis in approximately 20% of patients.

Commercially available enzyme immunoassays are improving and several algorithms have been advocated to resolve indeterminate tests. In deciding whom to test, clinicians should keep in mind that undiagnosed

patients result in most new transmission and that studies in many settings show that most patients welcome the opportunity to learn their serological status.

IgG antibodies are commonly negative in primary genital herpes since they become detectable 2 weeks to 3 months after the onset of symptoms and persist indefinitely. Thus, directly after infection there is a "window" in which testing for antibodies will give a negative result. Consequently, primary HSV infections can be documented by using any serologic methods to show seroconversion with paired sera. As IgM testing also can be positive during reactivation of disease, it cannot be used to distinguish primary from recurrent infection and, therefore, is of limited use for routine diagnostic purposes.

If genital lesions are present, type-specific serology and direct virus testing can help to establish if the episode is new acquisition of HSV infection or reactivation (Table 9.4). Type-specific HSV antibodies can take from 2 weeks to 3 months to develop. Thus, in a person with newly acquired herpes, the initial absence of antibodies to glycoprotein G2 (gG2) and subsequent development of such antibodies after 12 weeks confirms primary HSV-2 infection. The distinction between newly acquired HSV and reactivated HSV is helpful for epidemiological studies, and is sometimes helpful clinically for management of psychosocial issues.

Because nearly all HSV-2 infections are sexually acquired, the presence of type-specific HSV-2 antibody implies anogenital infection and education and counselling appropriate for persons with genital herpes should be provided. The presence of HSV-1 antibody alone is more difficult to interpret. The majority of persons with HSV-1 antibody have oral HSV infection acquired during childhood, which might be asymptomatic. However, acquisition of genital HSV-1 is increasing and genital HSV-1 may also be asymptomatic. Lack of symptoms in an HSV-1 seropositive person does not distinguish anogenital from orolabial infection. Persons with HSV-1 infection, regardless of site of infection, remain at risk for HSV-2 acquisition.

Type-specific glycoprotein G (gG)-based serological testing can distinguish HSV-1 infection from HSV-2 infection and should always be specifically requested when serology is performed. Type-specific HSV serologic assays may prove useful in the following situations: recurrent genital symptoms or atypical symptoms with negative HSV cultures; clinical diagnosis of genital herpes without laboratory confirmation; in partners of patients with proven genital herpes to show serological concordance or discordance prior to counselling. In addition, HSV serologic testing should be included in a comprehensive evaluation for STIs among persons with multiple sex partners and/or HIV infection, and among men who have sex with men at increased risk for HIV acquisition.

HSV-1 and HSV-2 genomes each encode at least 80 different structural and non-structural polypeptides, including at least 10 glycosylated proteins, designated glycoprotein A through glycoprotein I (gA–gI). The majority of the antibody to HSV infection is raised against these surface glycoproteins. Glycoprotein gB, gC, gD, and gE trigger potent immune responses. Some epitopes that are present on these glycoproteins are shared by HSV-1 and HSV-2 and, therefore, cause a significant degree of cross-reactivity when testing with certain commercial assays. However, no cross-reactivity between gG1 in HSV-1 and gG2 in HSV-2 has been detected (10). Accurate type-specific HSV serologic assays are based on the detection of HSV-specific gG1 (HSV-1) and gG2 (HSV-2) antibodies using native, purified, or recombinant gG1 or gG2 as antigens.

Type-specific HSV gG-based serologic ELISAs became commercially available in 1999, but older assays that do not accurately distinguish HSV-1 from HSV-2 antibody (despite claims to the contrary) remain on the market. POC rapid tests also can provide results for HSV-2 antibodies from capillary blood or serum during a clinic visit. The sensitivities of these gG type-specific tests for the detection of HSV-2 antibody vary from 80–98%, and false-negative results might be more frequent at early stages of infection. The specificities of these assays are ≥96%. The tests approved for use in the USA have a sensitivity of 97–100% and specificity of 94–98%, respectively in comparison to western blot (WB). False-positive results may occur, especially in patients with a low likelihood of HSV infection. Repeat or confirmatory

testing might be indicated in some settings, especially if recent acquisition of genital herpes is suspected.

Concurrent STIs may increase false–positive test results by HSV-2 ELISA (*11*). In addition, comparisons of ELISA-based tests to WB in sub-Saharan Africa demonstrate a lower specificity than previously observed, particularly in HIV-infected persons (*12*). More generally, the poor specificity of certain herpes type-specific gG-based tests has been reported on samples from sub-Saharan Africa (*12–14*). This reduced performance is not well understood but may result from laboratory/technical issues or cross-reactivity with unidentified infections more common in sub-Saharan Africa (*13, 15–16*).

Rapid POC serologic assays also have been developed for detection of HSV-2-specific antibodies. These immunoassays are designed to use capillary blood from a finger stick (or serum) and typically employ lateral flow of serum through a membrane containing a dot of gG1 or gG2 antigen. When serum is applied to the device, a visual colour change develops (pink dot) if HSV antibodies are present. Despite a reported inter–operative variability of 5–10% in test interpretation, these POC tests perform relatively well with sensitivities ≥91% and specificities ≥94% (*17*). In common with ELISA-based systems, the performance of POC systems depends in part on HSV prevalence within the population. The major benefit of POC assays is that they provide a result rapidly (potentially while the patient is still at the clinical site), allowing for more timely patient education and counselling. The major drawback of these tests is their high cost relative to ELISA-based systems.

HSV-specific IgG and IgM antibodies can be detected by several immunological methods (*18–19*). A number of non-commercial WBs have been developed in specialized reference laboratories, but they remain inaccessible for diagnostic use in most settings (*20*). Table 9.4 summarizes serologic diagnosis of HSV infections and HSV type-specific antibody testing.

Table 9.4: Virological and serological approach to HSV–2 diagnosis in the presence and absence of genital lesions, adapted from Gupta and colleagues (*9*)

	HSV-2 detection by direct method	HSV-1-specific IgG	HSV-2-specific IgG	Interpretation
First assessment of genital lesions	Positive	Positive or negative	Negative	• Acute HSV-2 infection • Repeat HSV-2-specific serology within 15–30 days
	Positive	Positive or negative	Positive	• Recurrent HSV-2 infection with HSV-2 infection acquired at least 6 weeks ago
No lesions	NA	Negative	Negative	• Patients at risk for acquiring orolabial or genital HSV-1 infection and/or HSV-2 infections
	NA	Positive	Negative	• Patients at risk for acquiring orolabial or genital HSV-2 infections
	NA	Positive	Positive	• HSV-1 and HSV-2 past-infections
Recurrent genital lesions	Positive	Positive or negative	Positive	• Recurrent HSV-2 infection
	Negative	Negative	Positive	• Possible recurrent HSV-2 infection • Other potential causes of genital ulcerative disease should be considered

NA, Not applicable.

Serological testing for HSV is not routinely recommended in asymptomatic patients, but is indicated in the following groups:

- History of recurrent or atypical genital disease when direct virus detection methods have been negative. HSV-2 antibodies are supportive of a diagnosis of genital herpes; HSV-1 antibodies do not differentiate between genital and oropharyngeal infection. Counselling of HSV-2 IgG-negative; HSV-1 IgG-positive patients should take into account that HSV-1 is a relatively uncommon cause of recurrent genital disease.

- First-episode genital herpes, where differentiating between primary and established infection guides counselling and management. At the onset of symptoms, the absence of HSV IgG against the virus type detected in the genital lesion is consistent with a primary infection. Seroconversion should be demonstrated at follow-up.

- Sexual partners of patients with genital herpes, where concerns are raised about transmission. Serodiscordant couples can be counselled about strategies to reduce the risk of infection and disease.

HSV serology and pregnancy:

- Testing of asymptomatic pregnant women is not routinely recommended, but is indicated when there is a history of genital herpes in the partner.

- HSV-1 and/or HSV-2 seronegative women should be counselled about strategies to prevent a new infection with either virus type during pregnancy.

HSV serology in the context of HIV infection:

- Testing of HIV-infected patients is not routinely recommended. Although HSV-2 seropositivity increases the risk of HIV transmission and frequent HSV recurrences augment HIV replication, there is limited evidence to inform the management of HSV-2 coinfection in HIV-infected patients without symptoms of genital herpes.

- Limited data suggest an increased risk of perinatal HIV transmission among HSV-2 seropositive HIV-infected women. As evidence is not consistent, testing of HIV-positive pregnant women is not routinely recommended.

- HSV-2 carriers who engage in high-risk sexual behavior should be counselled about the increased risk for HIV acquisition.

9.7 Therapeutic monitoring: Drug resistance testing

Long-term prophylaxis and treatment with antiherpetic drugs such as acyclovir or valacyclovir can result in the development of resistance, especially in immuno-compromised patients (*21*). The relative prevalence of acyclovir-resistant HSV isolates differs between immunocompetent and immunocompromised individuals on account of prolonged viral replication and impaired host response that may favour the survival of less fit drug-resistant HSV strains. The persistence of lesions for more than 1 week after the beginning of therapy without appreciable decrease in size, an atypical appearance of the lesions, or the emergence of new satellite lesions despite antiviral administration is suggestive of treatment failure. Laboratory diagnosis of acyclovir resistance is required to guide clinicians towards different treatment options in cases of therapy failure. Acyclovir resistance can be diagnosed by testing a virus against antiviral agents (phenotypic assays) or by the identification of specific thymidine kinase (UL23) and DNA polymerase (UL30) genes mutations conferring resistance to antiviral drugs (genotypic assays). For genotypic methods to be helpful in clinical practice, it is essential to be able to discriminate between random variations (polymorphisms) and true drug-resistant mutations.

9.8 References

1. Domeika M et al. Guidelines for the laboratory diagnosis of genital herpes in eastern European countries. *Eurosurveillance*, 2010, 15(44):pii:19703.

2. Centers for Diseases Control, Sexually transmitted diseases treatment guidelines 2010. *Morbidity and Mortality Weekly Report*, 2010, 59(RR–12) (http://www.cdc.gov/std/treatment/2010/STD-Treatment-2010-RR5912.pdf, accessed 4 April 2013).

3. Hobson A et al. Evaluation of a quantitative competitive PCR assay for measuring herpes simplex virus DNA content in genital tract secretions. *Journal of Clinical Microbiology*, 1997, 35(3):548–552.

4. Mbopi–Kéou FX et al. Interactions between herpes simplex virus type 2 and human immunodeficiency virus type 1 infection in African women: opportunities for intervention. *Journal of Infectious Diseases*, 2000, 182(4):1090–1096.

5. Ryncarz AJ et al. Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. *Journal of Clinical Microbiology*, 1999, 37(6):1941–1947.

6. Espy MJ et al. Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. *Journal of Clinical Microbiology*, 2000, 38(2):795–799.

7. Burrows J et al. Detection and subtyping of herpes simplex virus in clinical samples by LightCycler PCR, enzyme immunoassay and cell culture. *BMC Microbiology*, 2002, 2(1):12.

8. Legoff J et al. Real-time PCR quantification of genital shedding of herpes simplex virus (HSV) and human immunodeficiency virus (HIV) in women coinfected with HSV and HIV. *Journal of Clinical Microbiology*, 2006, 44(2):423–432.

9. Gupta R, Warren T, Wald A. Genital herpes. *The Lancet*, 2007, 370(9605):2127–2137.

10. Görander S, Svennerholm B, Liljeqvist JA. Secreted portion of glycoprotein g of herpes simplex virus type 2 is a novel antigen for type-discriminating serology. *Journal of Clinical Microbiology*, 2003, 41(8):3681–3686.

11. Summerton J et al. Effect of sexually transmitted disease (STD) coinfections on performance of three commercially available immunosorbent assays used for detection of herpes simplex virus type 2-specific antibody in men attending Baltimore, Maryland, STD clinics. *Clinical and Vaccine Immunology*, 2007, 14(12):1545–1549.

12. Biraro S et al. Performance of commercial herpes simplex virus type-2 antibody tests using serum samples from sub-Saharan Africa: a systematic review and meta-analysis. *Sexually Transmitted Diseases*, 2011, 38(2):140–147.

13. van Dyck E et al. Performance of commercially available enzyme immunoassays for detection of antibodies against herpes simplex virus type 2 in African populations. *Journal of Clinical Microbiology*, 2004, 42(7):2961–2965.

14. LeGoff J et al. Performance of HerpeSelect and Kalon assays in detection of antibodies to herpes simplex virus type 2. *Journal of Clinical Microbiology*, 2008, 46(6):1914–1918.

15. Ashley-Morrow R et al. Performance of focus ELISA tests for herpes simplex virus type 1 (HSV-1) and HSV-2 antibodies among women in ten diverse geographical locations. *Clinical Microbiology and Infection*, 2004, 10(6):530–536.

16. Golden MR et al. Herpes simplex virus type 2 (HSV-2) western blot confirmatory testing among men testing positive for HSV-2 using the focus enzyme-linked immunosorbent assay in a sexually transmitted disease clinic. *Sexually Transmitted Diseases*, 2005, 32(12):771–777.

17. Wald A, Ashley–Morrow R. Serological testing for herpes simplex virus (HSV)-1 and HSV-2 infection. *Clinical Infectious Diseases*, 2002, 35(Suppl 2):S173–S182.

18. Ashley RL et al. Premarket evaluation of a commercial glycoprotein G-based enzyme immunoassay for herpes simplex virus type-specific antibodies. *Journal of Clinical Microbiology*, 1998, 36(1):294–295.

19. Morrow RA, Friedrich D, Krantz E. Performance of the Focus and Kalon enzyme-linked immunosorbent assays for antibodies to herpes simplex virus type 2 glycoprotein G in culture-documented cases of genital herpes. *Journal of Clinical Microbiology*, 2003, 41(11):5212–5214.

20. Ashley RL. Sorting out the new HSV type specific antibody tests. *Sexually Transmitted Infections*, 2001, 77(4):232–237.

21. Piret J, Boivin G. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. *Antimicrobial Agents and Chemotherapy*, 2011, 55(2):459–472.

Chapter 10

Syphilis

10.1 Introduction

Syphilis is a chronic sexually transmitted disease characterized by florid manifestations and long periods of quiescence. It is caused by *Treponema pallidum subsp. pallidum*, a delicate spiral organism closely related to the causative organisms of the nonvenereal treponematoses, namely *T. pallidum subsp. pertenue* (yaws), *T. pallidum subsp. endemicum* (endemic syphilis), and *T. carateum* (pinta). These four pathogens are morphologically and antigenically identical. They can be differentiated only by their mode of transmission, epidemiology, clinical manifestations, and more recently, as a result of genetic sequencing (*1*).

Venereal syphilis usually is transmitted as a result of sexual contact with an infectious lesion of the mucous membranes or abraded skin, or transplacentally from a pregnant woman to her fetus. Bacterial multiplication occurs preferentially at the point of inoculation, resulting in the formation of a primary genital ulceration following an incubation period of 9–90 days. However, syphilis should be considered a systemic disease since the causative bacterium enters the bloodstream very soon after infection.

Figure 10.1 shows the course of untreated syphilis. The first manifestation of the disease in adults is a small macule, which becomes a papule, which in turn ulcerates. The typical ulcer (primary chancre) is classically a single, painless lesion that has a clean base and is relatively avascular. The lesion is commonly found in the coronal sulcus, on the glans, or penile shaft in men, and on the vulva, vaginal walls, or cervix in women. Extragenital lesions are rare, but oral chancres may occur as a result of fellatio, and perianal and rectal lesions are frequently seen in men who have sex with men or in women who have practiced anal-receptive intercourse. Since they are frequently painless, primary lesions may remain unnoticed. If left untreated, the ulcer will resolve spontaneously within 3–8 weeks without leaving a scar. Genital primary chancres usually are associated with a bilateral inguinal lymphadenopathy, which is classically discrete and non-tender.

In the untreated patient, the onset of the secondary stage of disease may occur some 6 weeks to 6 months after initial infection. The primary chancre may still be present when clinically apparent secondary lesions
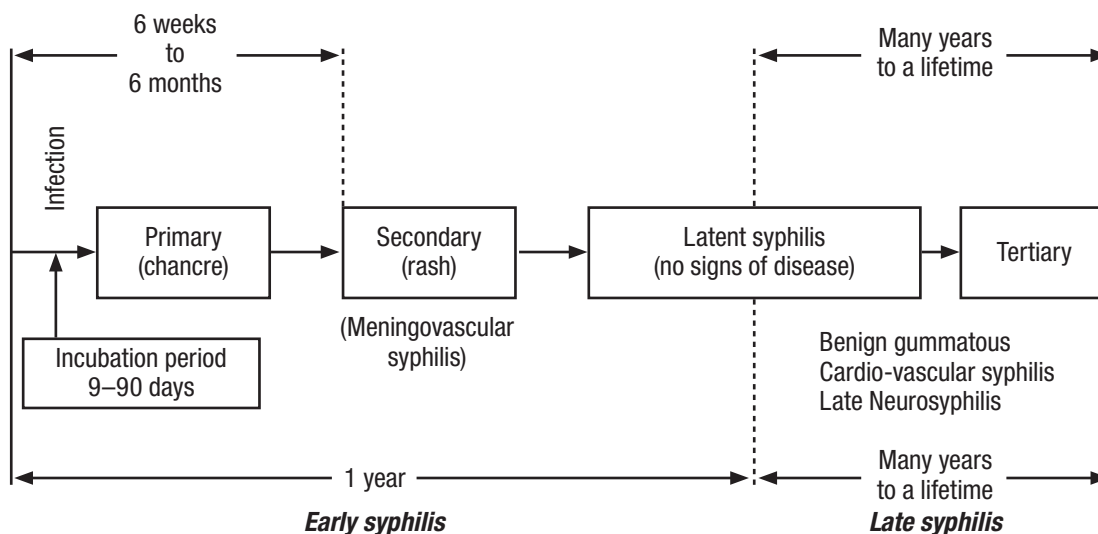

Figure 10.1
Schematic representation of the course of untreated syphilis

occur. The main feature of secondary syphilis is an evenly distributed, non-irritant skin rash that may be macular, papular or papulo-squamous; it often may be seen on the palms of the hands and soles of the feet. The rash may be accompanied by a generalized lymphadenopathy and fever, headache, and general malaise. In warm, moist areas such as the vulva or the perianal region, the rash may become enlarged to form elevated wart-like structures known as condylomata lata and on mucous surfaces form superficial grey-white serpiginous lesions known as "snail-track ulcers".

If secondary syphilis remains undiagnosed and therefore untreated, all visible manifestations of the disease resolve spontaneously and the patient will pass into a period of latency that may last many years. Latent syphilis conveniently is divided into early latent and late latent infection, with the dividing line being drawn 1 year after acquisition of the disease (see Fig.10.1). However, it should be borne in mind that it is often impossible to determine the exact duration of untreated infection and such cases should be classified as late latent disease by default. During the latent stages of the disease, there are no skin or mucous membrane lesions to sample; therefore, a diagnosis has to be based on the results of serological testing and the absence of signs and symptoms of tertiary syphilis.

Tertiary syphilis generally is considered to be the destructive stage of the disease. In general, the signs and symptoms of these late manifestations usually occur many years after the initial infection, although the disease process may proceed significantly more rapidly in patients coinfected with human immunodeficiency virus (HIV) infection. The various manifestations of tertiary syphilis have been classified as benign gummatous disease, cardiovascular syphilis, and neurosyphilis. However, these clinical features may coexist. Gummas are the destructive lesions of tertiary syphilis and can occur in any organ of the body—but most frequently in skin, cartilage, and bone (benign gummatous disease); the walls of the aorta (cardiovascular syphilis); the cerebral vessels (meningovascular syphilis); or the brain and spinal cord (neurosyphilis).

These late manifestations usually are diagnosed on clinical grounds combined with the results of chest X-ray or other imaging (for cardiovascular syphilis), X-ray examination of affected bones to detect bony gummas, and serological testing, including examination of cerebrospinal fluid for neurosyphilis. A more comprehensive description of the various clinical manifestations of the disease can be found in authoritative texts (2).

10.2 Direct detection methods for the diagnosis of syphilis

Because *T. pallidum* cannot be cultivated on artificial media, direct detection methods such as dark-field microscopy, direct immunofluorescence (IF), and tests to detect *T. pallidum*-specific DNA sequences in specimens obtained from skin lesions or tissues are the methods of choice to diagnose early syphilis. The rabbit infectivity test (RIT) has long been considered the "gold standard" for the direct detection of *T. pallidum* in clinical specimens (3). This method has a test sensitivity of nearly a single organism when repeated passages in rabbits are used. However, this test is rarely performed, except in research laboratories, since is time–consuming (requiring approximately 1–2 months to complete) and requires access to a suitable animal facility.

10.2.1 Dark-field microscopy

Dark-field microscopy is the only point-of-care (POC) method that is capable of establishing a direct diagnosis of syphilis in cases of adult primary or secondary or early congenital disease. Its use is therefore recommended for dedicated STI clinics and hospital laboratories close to the clinical site. Since a specialized microscope fitted with a dark-field condenser is required, together with adherence to strict technical conditions in order to produce reliable results, the test should be limited to specialist laboratories.

In dark-field microscopy, only light rays striking organisms or particles at an oblique angle enter the microscope objective, giving rise to bright, white luminescent bodies against a black background. Dark-field microscopy has to be performed by well-trained and experienced personnel who are able to adjust the microscope correctly and to differentiate *T. pallidum* from non-pathogenic treponemes and other spiral organisms commonly found on genital and anal mucous

membranes. As the oral cavity often is colonized by spirochaetes other than treponemes, dark-field examination of material from oral lesions is not recommended.

Both primary and secondary lesions of syphilis can be examined by dark-field microscopy. The ideal specimen is a serous exudate from active lesions, which is free of red blood cells. Active lesions should be cleansed carefully with a sterile gauze swab and sterile saline. The lesion then should be gently abraded with a sterile, dry gauze swab and squeezed to produce a serous exudate. If bleeding occurs, the drops of blood should be wiped away and the serous liquid that appears transferred to a glass slide using a thin stainless steel or platinum spatula or bacteriological loop, or by pressing the slide directly onto the fluid. The material may be mixed with a drop of saline to give a homogeneous suspension that then can be covered with a coverslip. The slide should be examined immediately, as the characteristic motility of the organisms is an important factor for identification. Any delay in examination rapidly reduces this motility. The chance of visualizing treponemes also decreases if the lesion is dry or is healing.

Correct optical alignment of the dark-field microscope is critical for successful dark-field microscopy. A few drops of immersion oil should be placed on the condenser of a previously aligned microscope. The condenser should then be lowered slightly so that the oil is below the level of the stage. The specimen to be examined then should be placed on the stage and the condenser elevated until there is good contact between the oil and the underside of the slide. It is important to avoid trapping air bubbles in the oil. The specimen should be initially examined using a low-power objective (10×).

After focusing the objective, the light should be centred in the middle of the field by adjusting the centring screws located on the condenser, and the condenser focused by rising and lowering the condenser until the smallest possible diameter of light is obtained. The light then should be re-centred if necessary. Using the dry 40× objective, the specimen should be brought into focus, and the slide examined carefully. Dark-field microscopy is best conducted in a darkened room.

T. pallidum appears as bright, white spiral bodies illuminated against a black background. The organism is identified by its typical morphology, size, and movement. It is a thin (0.10–0.18 μm wide) organism, 6–20 μm long, with 8–14 regular, tightly wound deep spirals (Fig. 10.2). It exhibits quick and rather abrupt movements. It rotates relatively slowly about its longitudinal axis. This rotation is accompanied by syncopated bending or twisting in the middle of the organism. Lengthening and shortening (like an expanding spring) may be observed. Distortion may occur in tortuous convolutions. Other, usually saprophytic, spirochaetes may be seen. However, they are often more loosely coiled or thicker and coarse with different movements, including a writhing motion with marked flexion and relaxation of the coils.

The demonstration of treponemes with the characteristic morphology and motility of *T. pallidum* constitutes a positive diagnosis for primary and secondary syphilis (*4*). Patients with a primary chancre that is dark-field-positive may be serologically negative, but normally would be expected to seroconvert within a few days. However, failure to visualize the organism on dark-field examination does not exclude a diagnosis of syphilis. Negative results may mean:

- the number of organisms present in the specimen is insufficient (a single dark-field examination has a sensitivity of less than 50%);

- the patient already has been treated or a topical antibacterial preparation has been applied to the lesion;

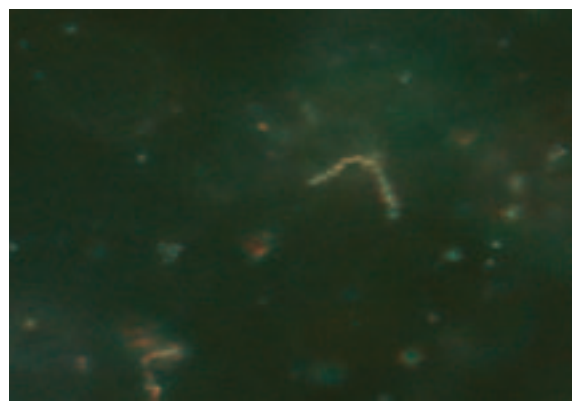- the lesion is approaching natural resolution;

- the lesion is not syphilitic.

Figure 10.2
Treponema pallidum, **dark-field microscopy**
Source: Courtesy of David Cox, Centers for Disease Control and Prevention, Atlanta, GA, USA.

Whatever the result of the dark-field examination, blood should always be taken for serological testing.

> NOTE: The dark-field technique can be practised and the microscope optics optimized by using specimens obtained along the gum margin, inside the mouth. Gingival epithelial cells and oral bacteria, including spiral organisms can be seen as bright, white bodies against a black background.

10.2.2 Direct fluorescent antibody (DFA) test

The method used to collect lesion material for the DFA test is identical to that used for dark-field microscopy. Specimens should be smeared onto a 1 cm^2 area of a microscope slide, allowed to dry in air, and fixed with acetone or methanol, after which they can be packed for transport to the laboratory. After adding commercially obtained fluorescein-labelled anti-*T. pallidum* globulin, incubation, and washing, the slides should be examined with a fluorescence microscope. Any *T. pallidum* spirochaetes in the specimen appear as apple-green-stained organisms with the typical *T. pallidum* morphology against a black background (Fig. 10.3). Both the specificity and sensitivity of the DFA test are superior to that of dark-field microscopy, especially if monoclonal antibody is used to make the fluorescein conjugate, because the DFA technique eliminates confusion with other spiral organisms and a small number of fluorescein-stained treponemes are more easily detected
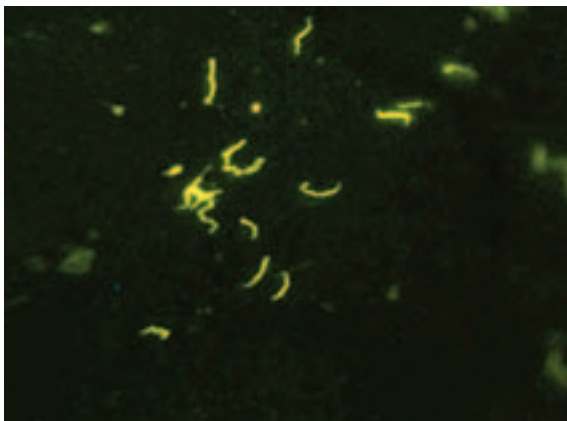

Figure 10.3
T. pallidum-positive direct immunofluorescence test
Source: Courtesy of David Cox, Centers for Disease Control and Prevention, Atlanta, GA, USA.

in the stained smear than in the unstained preparation (*5*). Unfortunately, the specific fluorescein conjugate is not commercially available in many countries.

> NOTE: The fluorescein conjugate can be conveniently titrated by applying twofold dilutions of the conjugate to commercial slides that can be purchased to perform the fluorescent treponemal antibody absorption (FTA-Abs) test, incubating, washing, and examining the specimen as in the direct immunofluorescence test procedure above. The dilution to be used for the direct immunofluorescence test is the highest dilution of conjugate that shows clear, specific fluorescence in the absence of background staining.

10.2.3 Nucleic acid amplification tests for *T. pallidum*

The polymerase chain reaction (PCR) test can detect DNA equivalents of <10 organisms in a specimen, by amplifying specific gene segments from *T. pallidum* genomic DNA. It can be used to examine specimens from any lesion exudate, tissue, or body fluid, and the specimen can be fresh, frozen, or fixed and paraffin-embedded. Because commercial PCR tests for *T. pallidum* cleared by the United States of America Food and Drug Administration are unavailable, some laboratories have implemented laboratory-developed PCR tests.

Several PCR assays have been developed and have been successfully used to detect to detect *T. pallidum*-specific DNA target sequences in primary and secondary lesions (*6–8*). The analytical sensitivity of these assays is approximately 10 organism equivalents. Use of fluorescein-labelled primers and an ABI 310 Genetic Analyzer to detect the amplicons has improved the analytical sensitivity of a *polA* PCR to approximately one organism per PCR reaction. These methods theoretically can be applied to the diagnosis of congenital syphilis and also neurosyphilis where the number of organisms is likely to be low; however, the tests cannot be recommended for the routine detection of *T. pallidum* in blood—even in primary and secondary disease owing to the presence of PCR inhibitors. However, a real-time,

semiquantitative PCR assay was utilized to indicate that the number of spirochetes in the blood of patients varies by stage of syphilis, ranging from 200 to 10^5 organisms per ml of blood (*9*).

Multiplex PCR assays have been developed for the simultaneous detection of the most common causes of sexually transmitted genital ulcer disease (GUD), namely *T. pallidum*, *Haemophilus ducreyi*, and herpes simplex virus (*10*). The *T. pallidum* target in the multiplex PCR is the 47–kDa gene, and a capture enzyme-linked immunosorbent assay (ELISA) is used to detect specific amplicons. This assay has been adapted to a real-time multiplex format that has been used to determine causes of genital ulceration in a number of different country settings and subsequently to determine appropriate syndromic algorithms for the management of GUD.

10.3 Serological testing for syphilis

Serological tests for syphilis conveniently may be divided into two types: the non-treponemal or reagin tests such as the Wasserman reaction (WR), rapid plasma reagin (RPR), Venereal Disease Research Laboratory (VDRL), and toluidine red unheated serum test (TRUST) tests; and the treponemal tests such as the FTA-Abs, *T. pallidum* haemagglutination assay (TPHA), *T. pallidum* passive particle agglutination assay (TPPA), ELISA, chemiluminescence, and the vast majority of POC or rapid tests that are commercially available at the present time.

10.3.1 Non-treponemal serological tests

All the current non-treponemal serological tests for syphilis detect reagin (a mixture of IgG and IgM antibodies in the sera of patients with syphilis) that is capable of reacting with a complex antigen (a mixture of cardiolipin, lecithin, and cholesterol) in the tests. The earliest non-treponemal serological test using this antigen was the WR test that is based on the principle of complement fixation. However, the most frequently used tests are based on flocculation reactions that may or may not include indicator particles. It is thought that anti-lipid IgG and IgM antibodies are formed as part of the host response to material released from damaged host cells early in infection as well as to lipids from the cell surface of the causative organism (*11*, *12*).

Although they are very sensitive and can be quantified, the non-treponemal tests lack specificity for syphilis and false-positive reactions have been estimated to occur in 0.2–0.8% of tests. These are associated with various medical conditions unrelated to syphilis (*11*, *12*). Acute false-positive reactions (which persist for periods of fewer than 6 months) generally have been found to be associated with other infectious diseases such as malaria, hepatitis, chicken pox, or measles, or with recent vaccination. In contrast, chronic false-positive reactions (which persist for more than 6 months) have been found to be associated with connective tissue disorders, malignancies, chronic infections such as leprosy, intravenous drug abuse, and ageing. Therefore, theoretically, sera found to be reactive in non-treponemal tests should be confirmed with a more specific treponemal test.

It should be noted that, although pregnancy has long been considered a condition possibly associated with false-positive non-treponemal tests, the rate of erroneous positive tests in pregnant women appears to be no greater than in their non-pregnant counterparts and may be solely related to the large number of pregnant women tested for the disease, particularly in low-prevalence settings (*13*). As a rule, the vast majority of false-positive sera show antibody titres of ≤1:4. To exclude false-positive non-treponemal results, all sera found to be reactive in a non-treponemal test should be confirmed using a treponemal test.

However, low titres do not exclude syphilis and are often found in late latent and tertiary disease.

The determination of non-treponemal serum titres using a quantitative procedure may be helpful for more correct interpretation of results and for evaluation of patients after treatment.

When used as initial screening tests, non-treponemal tests become positive around 6 weeks after infection; therefore, up to 40% of dark-field or PCR-positive primary lesions may be initially seronegative. Following seroconversion, non-treponemal antibody titres rise to reach a peak between 1–2 years following infection—if no effective treatment is provided. Thereafter, during the late latent and tertiary stages of the disease, the titre will decline slowly, frequently becoming negative in very late

disease. The great advantage of non-treponemal tests is that they may be used to assess the efficacy of therapy. Thus, after successful treatment of early syphilis (i.e. primary, secondary, or early latent disease), the titre of non-treponemal antibody should fall significantly (i.e. at least a fourfold drop in titre) and eventually become negative. However, provision of successful treatment during later stages of the disease may result in persistent seropositivity albeit at a low titre (Fig. 10.4). It should be noted that any subsequent rise (fourfold or greater) in titre could indicate either relapse or reinfection.

10.3.1.1 Venereal Disease Research Laboratory (VDRL) test

In the VDRL test, the antigen is not stabilized and a suspension must be freshly prepared on the day of use. The test should be performed on heated (56°C) serum and the results are read with a microscope at 100× magnification. The VDRL remains the test of choice for the detection of reagin in cerebrospinal fluid (CSF)

specimens obtained from patients with suspected neurosyphilis. A detailed description of the VDRL test is outlined below. The unheated serum reagin test is an improved version of the VDRL test performed on unheated serum and using a stabilized antigen.

VDRL test procedure (adapted from Larsen et al., 1998; 3).

Reagents and equipment required:

1. VDRL antigen. A colourless, alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol, and 0.21% + 0.01% lecithin. The antigen should be stored either in the dark at room temperature (23–29°C) or refrigerated at 2–8°C, but not frozen. At these temperatures, the antigen components remain in solution. Bottles or vials that contain a precipitate should be discarded.

2. VDRL-buffered saline, pH 6.0 ± 0.1 (1.0% NaCl). VDRL-buffered saline may be purchased or prepared in the laboratory.
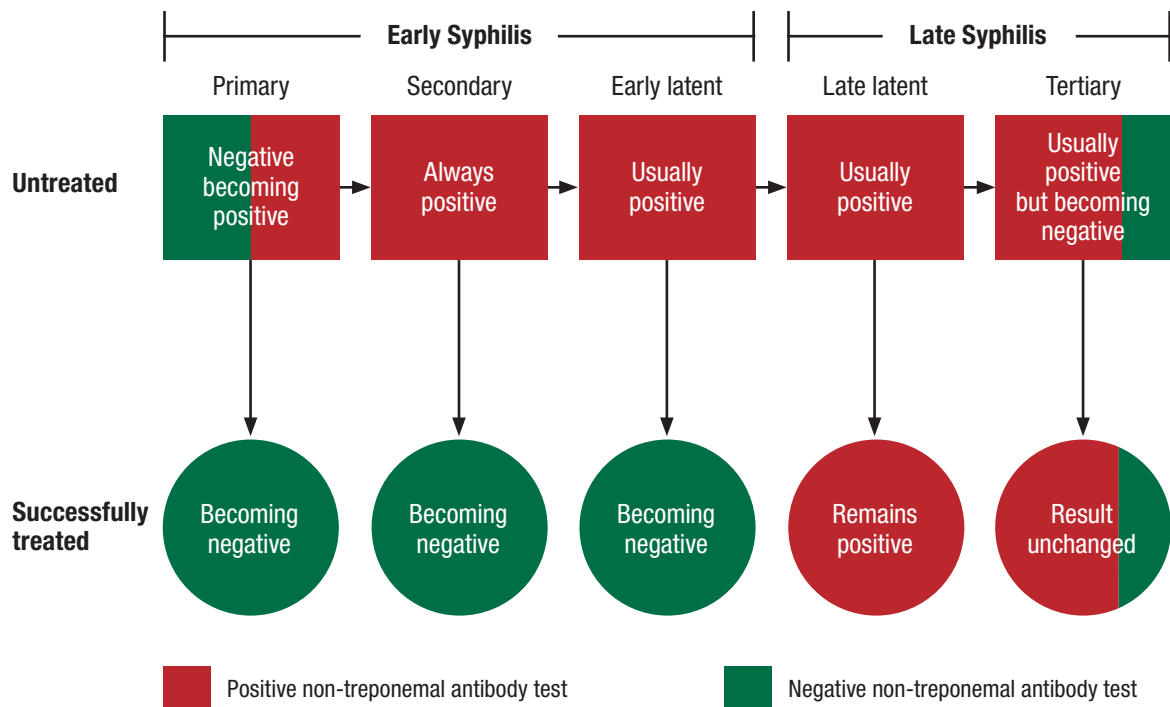

Figure 10.4
Reactivity of non-treponemal serological tests during the course of untreated syphilis (boxes) and response of tests following successful therapy (circles), by stage of disease

VDRL-buffered saline comprises:

Formaldehyde, neutral (ACS)	0.5 ml
Na_2HPO_4, anhydrous	0.037 g
KH_2PO_4	0.170 g
NaCl	10.00 g
Distilled water	1000.0 ml

The pH of the solution should be measured and the solution stored in screw-capped bottles.

Note: When an unexplained change in reactivity of the controls occurs, the pH of the saline should be measured to determine whether this is a factor. Buffered saline that is outside the range of pH 6.0 ±0.1 should be discarded.

3. Control serum samples. Reactive (R), weakly reactive (W), and nonreactive (N) sera in lyophilized or liquid form are used as controls in the test. If quantitative tests are to be performed, a control serum that can be titrated to at least a 1:4 dilution should be used.

4. Acetone

5. Alcohol, 95% ethanol

6. Paraffin

7. 0.9% saline. Add 0.9 g of dry sodium chloride to each 100 ml of distilled water.

8. 10.0% saline. Add 10 g of dry sodium chloride to each 100 ml of distilled water.

Equipment:

1. Non-disposable calibrated needles without bevels
   a. For serological test: 18-gauge
   b. For CSF test: 21- or 22-gauge

2. Bottles, 30 ml with flat inner-bottom surface.

3. Safety pipetting device with disposable tips delivering 50 µl.

4. Pipettes, 1.0 ml, 5.0 ml, and 10.0 ml.

5. Microscope slides, measuring 5 × 7.5 cm, with 12 paraffin or ceramic rings approximately 14 mm in diameter. Note: The rings must be high enough to prevent spillage during rotation.

6. Slide holder, for 5 × 7.5 cm slides.

7. Ringmaker, to make paraffin rings approximately 14 mm in diameter (Cat.# 2600, Eberbach Corp., Ann Arbor, MI, USA)

8. Mechanical rotator adjustable to 180±2 rpm, circumscribing a circle 19 mm in diameter on a horizontal plane.

9. Binocular microscope with 10× eyepieces, 10× objective.

10. Discard containers; disinfectants.

11. Disposable latex gloves, safety glasses, and protective clothing.

12. Cover for slides while on rotator to maintain humidity and prevent drying.

13. Syringes, 2 ml or 5 ml.

Test procedures for serum specimens

Preparing the antigen suspension:

1. Prepare fresh VDRL antigen suspension each day. Maintain the temperature of the buffered saline, antigen, and equipment between 23–29°C at the time the antigen suspension is prepared.

2. Dispense 0.4 ml of VDRL-buffered saline into the bottom of a round, 30-ml, stoppered bottle with a flat inner–bottom surface or a 25 ml stoppered flask.

3. Add 0.5 ml of VDRL antigen suspension directly onto the saline, while rotating the bottle continuously but gently on a flat surface. Antigen should be added drop by drop at a rate allowing approximately 6 seconds for each 0.5 ml of antigen.

4. The last drop of antigen should be expelled from the pipette without touching the pipette to the saline and rotation of the bottle continued for 10 seconds.

5. Add 4.1 ml of buffered saline.

6. Cap the bottle and shake approximately 30 times in 10 seconds. The antigen suspension is then ready for use and may be used during that working day.

7. Mix the VDRL antigen suspension by gently swirling it each time it is used. (The suspension should not be mixed by forcing it back and forth through a syringe and needle, since this may cause breakdown of particles and loss of reactivity.)

Qualitative test (serum):

1. Slide flocculation tests for syphilis are affected by room temperature. For reliable and reproducible test results, the VDRL antigen suspension, controls, and test specimens must be kept at room temperature, 23–29°C, when tests are performed.

2. Add 50 µl of the sera to be tested into each ring of a paraffin or ceramic-ringed slide.

3. Holding the VDRL antigen suspension-dispensing needle and syringe in a vertical position, dispense several drops of antigen to clear the needle of air. Then add one free-falling drop (17 µl) of antigen suspension to each ring containing serum.

4. Place the slide on the mechanical rotator and rotate the slide for 4 minutes at 180±2 rpm under a cover to maintain a humid atmosphere and prevent excessive evaporation.

5. Immediately after rotation, read the slide and record the test results.

6. All serum specimens that produce reactive, weakly reactive, or "rough" non-reactive results in the qualitative VDRL slide test should be tested quantitatively and the end-point titre recorded.

Reading and reporting results:

1. Read slides microscopically, using 10× eyepieces and a 10× objective.

2. Report results as follows:

Reading	*Report*
• Medium or large clumps	• Reactive (R)
• Small clumps	• Weakly reactive (W)
• No clumping or very slight roughness	• Nonreactive (N)

Quantitative test (serum):

1. Prepare two-fold dilutions of the serum to be titrated. Quantitative tests for three serum specimens up to the 1:8 dilution may be performed on one slide.

2. Perform the test on the two-fold dilutions of serum in exactly the same manner as for the qualitative test.

3. Read the results microscopically using 10× eyepieces and a 10× objective as for the qualitative test.

4. Record titres as the highest dilution that yields a reactive (not weakly reactive) result.

5. After completing the day's tests, discard the antigen suspension and clean the dispensing needle and syringe by rinsing with water, alcohol, and acetone, in that order. Remove the needle from the syringe after cleaning.

> NOTE: The VDRL test is the preferred test to be performed on CSF for the diagnosis of neurosyphilis. The test is performed in an identical manner to that when the test is conducted on serum; however, the CSF specimen does not have to be heated to 56°C prior to testing.

10.3.1.2 Rapid plasma reagin (RPR) test

The main advantages of the RPR over the VDRL include the use of a stabilized antigen, the use of cards instead of slides, and the addition of charcoal particles to the antigen as an indicator of flocculation. The antigen is not coated onto these particles, but the charcoal is trapped in the lattice formed by the antigen–antibody complex in reactive samples, causing the reaction to become visible to the naked eye. The test can be performed on unheated serum or plasma and is performed within 18 mm circles on plasticized cards. The RPR is the most widely available macroscopic non-treponemal test and is used around the world. Figure 10.5 shows the procedure for performing the RPR test.
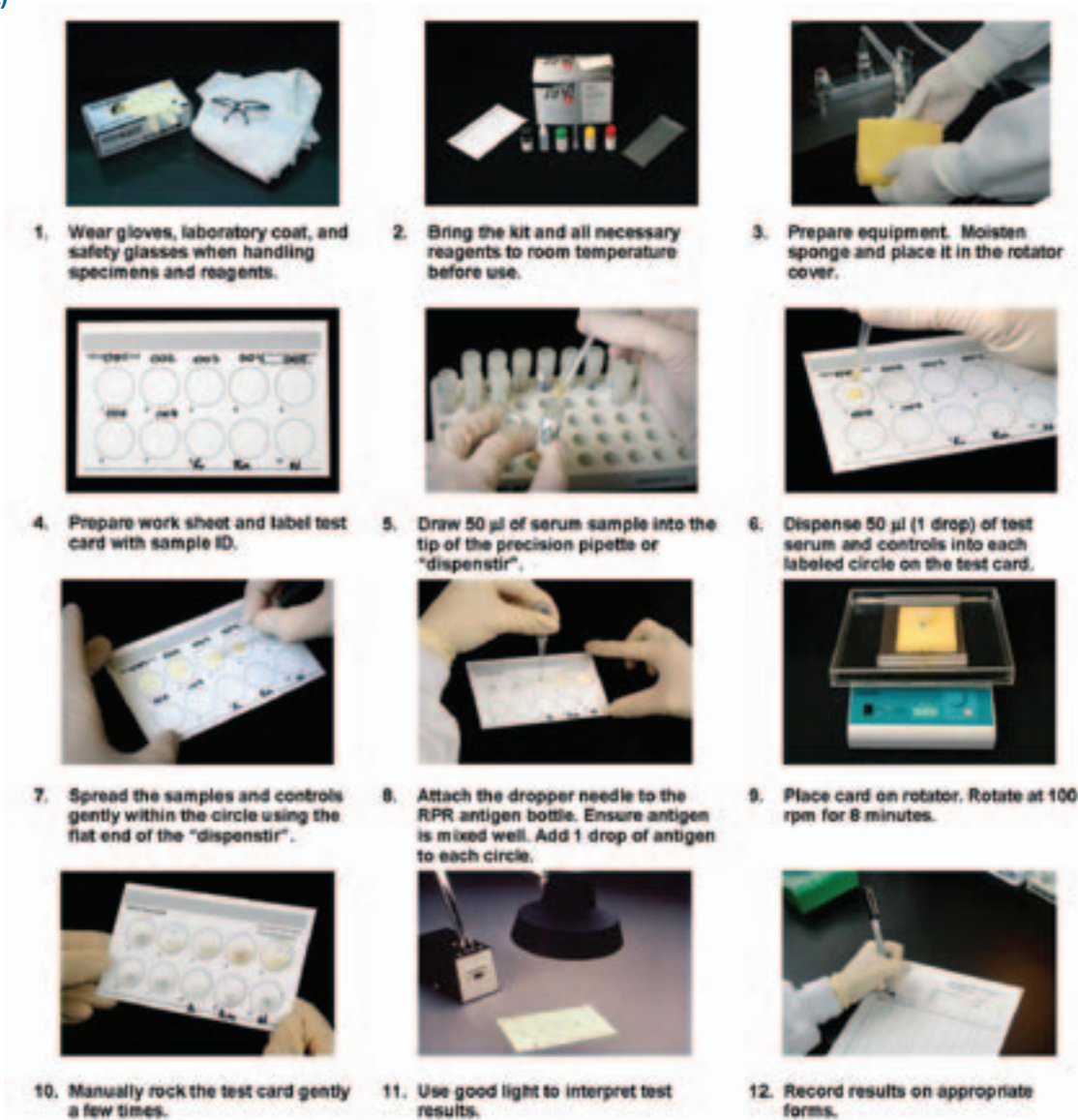
(A)

1. Wear gloves, laboratory coat, and safety glasses when handling specimens and reagents.

2. Bring the kit and all necessary reagents to room temperature before use.

3. Prepare equipment. Moisten sponge and place it in the rotator cover.

4. Prepare work sheet and label test card with sample ID.

5. Draw 50 µl of serum sample into the tip of the precision pipette or "dispenstir".

6. Dispense 50 µl (1 drop) of test serum and controls into each labeled circle on the test card.

7. Spread the samples and controls gently within the circle using the flat end of the "dispenstir".

8. Attach the dropper needle to the RPR antigen bottle. Ensure antigen is mixed well. Add 1 drop of antigen to each circle.

9. Place card on rotator. Rotate at 100 rpm for 8 minutes.

10. Manually rock the test card gently a few times.

11. Use good light to interpret test results.

12. Record results on appropriate forms.

Figure 10.5
Procedures for performing (A) and interpreting (B) the rapid plasma reagin (RPR) test
Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

A modification of the RPR, the TRUST uses toluidine red instead of charcoal to visualize the flocculation reaction. Unlike those used in the RPR test, TRUST reagents do not require refrigerated storage.

10.3.2 Treponemal serological tests

In contrast to the non-treponemal tests, the treponemal tests are considered to be more specific. However, rare false-positive treponemal results have been recorded, which may be transient and of unknown cause, or associated with connective tissue disorders (*11*). There
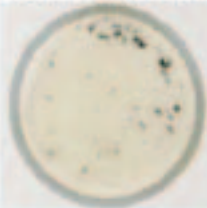
is little value in monitoring responses to therapy using treponemal tests since, after seroconversion, they usually remain positive for life—even after provision of successful therapy (see Fig 10.6). There is, therefore, no point in performing quantitative treponemal assays as part of diagnostic algorithms. Since some treponemal tests may become reactive prior to the non-treponemal tests (the FTA-Abs test may become reactive approximately 3 weeks after infection), some patients with very early primary infection may be non-treponemal test seronegative, treponemal test seropositive. However,

(B)

Qualitative Results :

Reactive
(Clumping/agglutination appears)

Minimally Reactive
(Slight clumping/agglutination)

Non-reactive
(Smooth gray pattern).

Samples giving the same or a higher degree of reactivity as the minimally reactive control (Rm) are reactive (R). The bottom 3 circles on the right-hand side of the card are the controls: reactive "R," minimally reactive "Rm," and nonreactive "N."

All Reactive samples should be tested by Quantitative RPR to obtain a titer level.

All Reactive samples should be tested by Quantitative RPR to obtain a titer level.

Report as Non-reactive .

Quantitative RPR Test Procedure:

1. Label a test card with sample IDs and 2-fold serial dilutions. Each sample will be tested undiluted (1:1) and diluted (1:2, 1:4, etc.)
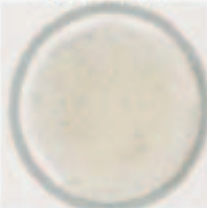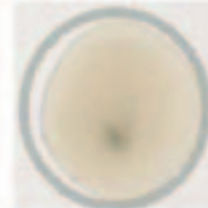
2. Add 50 μL of 0 .9% saline to each circle that will contain a diluted sample or control. Do NOT add saline to the first circle for each sample (1:1 well).

3. Add 50 μL of sample to the first (1:1) and second (1:2) circles.

4. Using a pipette, mix the specimen with the saline drop by drawing liquid up and down into the pipette tip 5-6 times.

5. Transfer 50 μL of this mixture (circle 2) to the next circle (3) and repeat mixing step until the last dilution is performed. Discard the last 50 μL.

6. Follow step 7-12 on page 1 (Qualitative RPR) and record the results such that the dilution level is recorded; ie. highest reactive dilution at 1:4 would be reported as R4.

Quantitative Results:

Report the highest reactive dilution. In the case above, specimen 7 (1st row) shows reactivity at the 1:1 dilution and is recorded as R1. Specimen 8 (2nd row) is an R4. The bottom 3 circles on the right-hand side of the card are the controls: reactive "R," minimally reactive "Rm," and nonreactive "N."

In the case above, the specimen is reactive until the 1:64 dilution and would be reported as an R64. If the highest dilution is still reactive, the specimen must be diluted further. Prepare a new test card and extend the dilutions out until the mixture in the circle is nonreactive.

Figure 10.5 (*continued*)
Procedures for performing (A) and interpreting (B) the rapid plasma reagin (RPR) test
Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

great care should be taken in interpreting the results of these serological tests, because the finding of a negative non-treponemal test and a positive treponemal test is most frequently an indication of previously treated early disease unless there are obvious signs of current primary infection (see and compare Figs 10.4 and 10.6).

All the current treponemal tests use whole cell lysates of *T. pallidum* or single or a mixture of recombinant treponemal antigens to detect antibodies against specific treponemal cellular components. There are a number of different test platforms used for treponemal serological testing, including indirect IF, agglutination
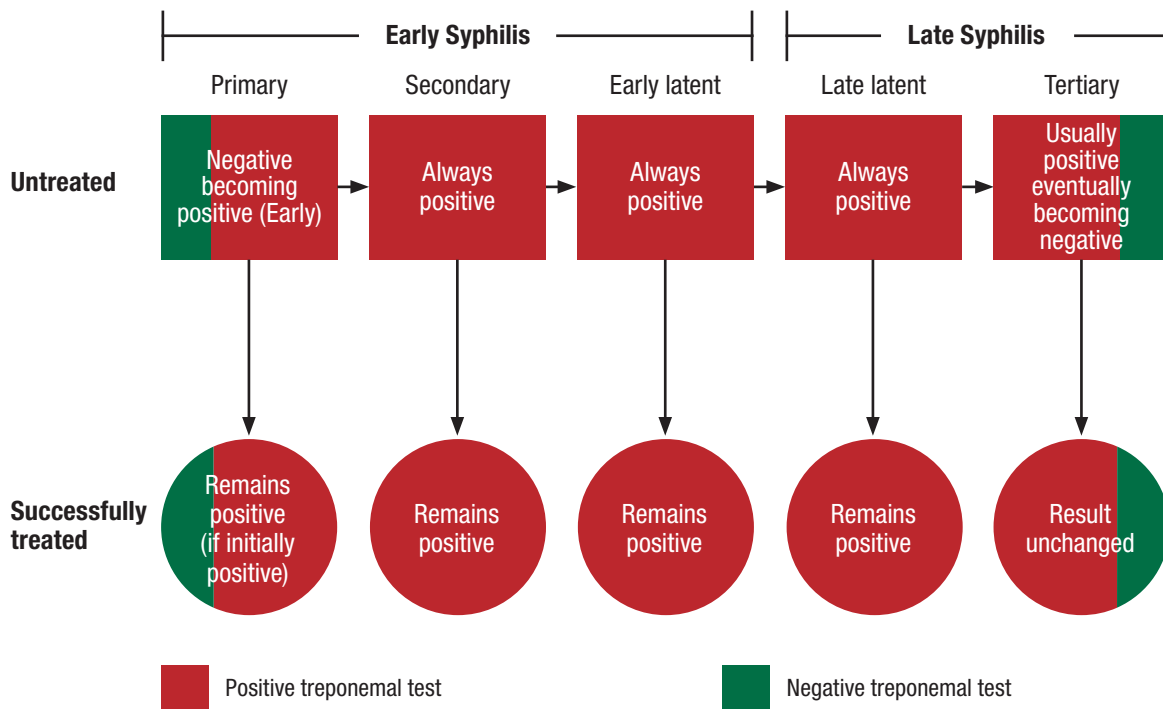
Figure 10.6
Reactivity of treponemal serological tests during the course of untreated syphilis (boxes) and response of tests following successful therapy (circles), by stage of disease.

testing using sensitized erythrocytes or gelatin particles, ELISAs, including variants that use chemiluminescence technology and immunochromatographic (lateral flow), or flow-through POC tests. All these assays are highly specific and were initially designed for use as confirmatory tests. However, those tests that are amenable to automation, namely some ELISA and chemiluminescence assays, have been licensed in some countries for use as initial screening tests, and treponemal POC tests often are used as the sole indicator of infection, particularly in resource-poor settings.

10.3.2.1 The Fluorescent Treponemal Antibody Absorption (FTA-Abs) test

The FTA-Abs test has long been considered the "gold standard" treponemal test, but it has recently been superseded by tests that are more sensitive, technically less demanding, and far less subjective. Both the performance of the test and the reading of the results have to be thoroughly checked. A good quality fluorescence microscope, experience in reading, quality
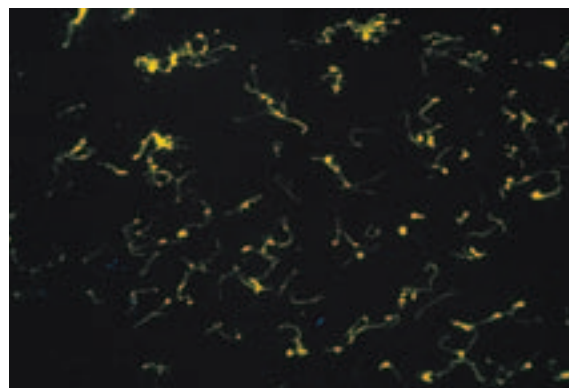
Figure 10.7
A positive FTA-Abs test showing fluorescing _T. pallidum_ spirochaetes
Source: Adapted from Larsen et al., 1998 (*3*).

reagents, and an appropriate dilution of the conjugate are all critical to the reliability of the test (Fig. 10.7). Unfortunately, both false-positive and false-negative FTA-Abs results are common due to laboratory error and subjective reading of the test (*14*, *15*). For the detailed method see below.

Reagents:

1. *T. pallidum* antigen slides can be obtained commercially. Alternatively, the antigen can be purchased as a suspension or prepared from *T. pallidum* (Nichols strain) extracted from rabbit testicular tissue and washed in phosphate-buffered saline (PBS) to remove rabbit globulin. Store unopened vials at 2–8°C.

2. Fluorescein isothiocyanate (FITC)-labelled antihuman immunoglobulin.

3. Prepare sorbent from cultures of nonpathogenic Reiter treponemes, usually with no preservative added. It is frequently dispensed in 5 ml amounts and lyophilized or as a suspension.

4. Reactive control serum. Obtain a pool of human serum from seropositive donors that are 4+ reactive. Dispense the pooled serum into aliquots and store frozen, preferably at –70°C or below. The highly reactive serum can be diluted appropriately with non-reactive sera to produce a minimally reactive 1+ control. The 1+ control displays the least degree of fluorescence reported as reactive and is used as a reading standard.

5. Non-specific control serum. The nonspecific control serum is a serum pool obtained from individuals without syphilis. No preservative is added. This control shows a >2+ nonspecific reactivity at a 1:5 dilution in PBS and essentially no staining when diluted 1:5 in sorbent.

6. A low fluorescence, non-drying immersion oil.

7. Acetone.

Reagents to be prepared:

1. Phosphate-buffered saline (PBS). Should be prepared by the following formulation in distilled water and stored in large volumes:

NaCl	7.65 g
Na_2HPO_4	0.724 g
KH_2PO_4	0.21 g
Distilled H_2O	1000 ml

   The pH should be determined and adjusted to pH 7.2 ± 0.1 with 1 N NaOH.

2. 2.0% Tween 80 in PBS.
   Warm the reagents in a 56°C water-bath. To 49 ml of sterile PBS, add 1 ml of Tween 80. Adjust the pH to 7.2 with 1 N NaOH. Discard the reagent if a precipitate develops or the pH changes.

3. Mounting medium. Add one part PBS, pH 7.2, to nine parts glycerine (reagent grade).

Equipment:

1. Incubator, 35–37°C

2. Waterbath, adjustable to 56°C

3. Centrifuge

4. Safety pipetting devices

5. Micropipettes delivering 10 µl to 200 µl

6. Loop, bacteriological, standard, 2-mm diameter, 26 gauge, platinum

7. Absorbent paper

8. Slide board with moist chamber and paper towels

9. Staining dishes, glass or plastic, with removable slide carriers

10. Microscope slides, 1 × 3 inches, with a frosted end, 1-mm thick, with 2 circles, 1-cm inside diameter

11. Coverslips, no. 1, 22-mm square

12. Test tubes (12 × 75 mm) and holders

13. Discard containers and disinfectants

14. Disposable latex gloves, safety glasses, and protective clothing

15. Fluorescence microscope with 10× eyepieces and 10× and 40× objectives

16. Vortex mixer.

If antigen slides are not acquired commercially, they can be prepared from treponemal suspensions as follows:

1. Wipe slides with clean gauze following storage of clean slides in alcohol.

2. If necessary, rehydrate the antigen according to manufacturer's instructions. Store opened vials at 2–8°C. These should be stable for a week.

3. Thoroughly mix antigen suspensions on a vortex mixer for 10 seconds and examine samples by dark-field microscopy to ensure that treponemes are adequately dispensed (single organisms rather than clumps) before making slides for the FTA-Abs test.

4. Prepare very thin *T. pallidum* antigen smears within each circle using a 2-mm wire loop. Place one loop-full of antigen within the two 1-cm circles and allow to air dry for at least 15 minutes.

5. Fix slides in acetone for 10 minutes and allow to air dry. Store acetone-fixed smears at –20°C. Smears should not be thawed and refrozen.

Sorbent:

Rehydrate freeze-dried sorbent with sterile distilled water or according to manufacturer's instructions. Store the rehydrated sorbent at 2–8°C or at –20°C. It is usable as long as acceptable reactivity is obtained and the product is not contaminated.

Fluorescein-labelled antihuman immunoglobulin (conjugate):

1. Rehydrate the FITC-labelled conjugate according to manufacturer's instructions. If cloudiness is observed, centrifuge at 500g for 10 minutes. Aliquot in small volumes and store at –20°C. The thawed conjugate should not be refrozen, but store at 2–8°C.

2. Prepare serial doubling dilutions of the new conjugate in PBS pH 7.2, containing 2% Tween 80 so that the dilutions include the titre suggested by the manufacturer.

3. Test each conjugate dilution with the reactive 4+ control serum diluted 1:5 in PBS, and with the appropriate minimally reactive 1+ control dilution using the FTA-Abs procedure described below.

4. Include a nonspecific staining control with each conjugate dilution.

5. Prepare a previously tested conjugate at its working dilution and perform tests with a reactive 4+ control serum, a minimally reactive 1+ control serum, and a nonspecific staining control with PBS to act as controls when first testing a new conjugate batch.

6. Read slides in the following order:

   a. Examine the three control slides (in 5 above) to ensure that reagents and testing conditions are satisfactory.

   b. Examine the new conjugate slides, starting with the lowest dilution of conjugate and record readings as 1+, 2+, 3+, or 4+.

   c. The endpoint of the titration is the highest dilution, giving maximum 4+ fluorescence with the reactive serum control and a 1+ reading with the 1+ dilution. The working titre of the new conjugate is one doubling dilution below the endpoint and should be the endpoint of the minimally reactive control.

   d. The new conjugate should not stain nonspecifically at three doubling dilutions below the working titre of the conjugate.

   e. Store the conjugate as directed by the manufacturer and dispense in not less than 0.3 ml aliquots at less than –20°C. A conjugate with a working dilution of 1:1000 or higher may be diluted 1:10 with sterile PBS containing 0.5% bovine serum albumin and 0.1% sodium azide prior to freezing.

   f. Verify the titre of the conjugate after storage for several days in the freezer.

Test procedure:

1. Identify previously prepared antigen slides by numbering the frosted end.

2. Number each tube and slide to correspond to the test serum and the control serum to be tested.

3. Prepare reactive (4+), minimally reactive (1+), and nonspecific control serum dilutions in sorbent or PBS according to the directions.

4. Pipette 200 µl of sorbent into a test tube for each test serum.

5. Add 50 µl of the heated test serum to the appropriate tube and mix.

6. Cover the appropriate antigen smears with 30 µl of the reactive (4+), minimally reactive (1+), and nonspecific control serum dilutions.

7. Cover the appropriate antigen smears with 30 µl of the PBS and 30 µl of the sorbent for nonspecific staining controls.

8. Cover the appropriate antigen smears with 30 µl of the test serum dilutions.

9. Prevent evaporation by placing slides in a moist chamber and incubate at 35–37°C for 30 minutes.

10. Place slides in slide carriers and rinse for 5 seconds with running PBS. Then place slides in staining dish containing PBS for 5 minutes and agitate slides by dipping them in and out of the PBS at least 20 times. Using fresh PBS, repeat the rinsing procedure once again. Finally, rinse slides for 5 seconds in running distilled water and gently blot with absorbent paper.

11. Dilute FITC-labelled antihuman IgG to its working titre in PBS containing 2% Tween 80 and layer approximately 30 µl of the diluted conjugate onto each smear.

12. Repeat steps 9 and 10.

13. Mount slides immediately by placing a small drop of mounting medium on each smear and applying a cover glass.

14. Place slides in a darkened room and read within 4 hours.

15. Check smear by dark-field microscopy, using the tungsten lamp first, to verify the presence of treponemes on the smear, then read using the fluorescence microscope using the appropriate FITC filters.

10.3.2.2 Treponemal agglutination assays

The TPHA and TPPA are easier to perform than the FTA-Abs test. Their sensitivity is similar to that of the FTA-Abs. These agglutination assays are also more practical than the FTA-Abs test for batch processing of large numbers of specimens. The TPHA, and more recently the TPPA has emerged as the confirmatory treponemal test of choice in many laboratories. Figure 10.8 shows the procedure for performing the TPPA test. The method for performing the TPHA is similar.

10.3.2.3 Treponemal enzyme immunoassays (EIAs) and chemiluminescence sssays (CIAs)

EIAs and CIAs for detecting antibody to *T. pallidum* are even more recent developments. Their sensitivity and specificity are comparable to those of FTA-Abs and agglutination assays.

The majority of treponemal EIAs employ either sonicated *T. pallidum* antigen, a single recombinant treponemal antigen, or a mixture of recombinants coated on to the wells of microtitre plates. A dilution of patient's serum is added to each well. If specific antibodies to *T. pallidum* are present in the serum, they will bind to the treponemal antigens. After washing off any excess antibody, a conjugate comprising biotinylated goat anti-human IgG labelled with streptavidin-peroxidase is added to detect bound-specific antibody. After a further washing step to remove any excess conjugate, an enzyme substrate is added to detect the antigen–antibody–conjugate complex. A colour reaction takes place if the patient has antibodies to the *T. pallidum* antigen(s). The intensity of the colour development is directly proportional to the concentration of antibodies present. The colour change is read using a plate reader.

In some EIAs, a different approach is taken to detection of specific antibodies. Specific recombinant treponemal 15-, 17-, 44.5-, and 47-kD antigens are immobilized on microplate wells. The patient's serum is then added to the wells, and if anti–treponemal antibodies are present they will specifically bind to the immobilized antigens. All non-bound proteins then are removed during a washing step. The same recombinant antigens that have been conjugated to horseradish peroxidase then are added to the wells of the plate. After a further washing step to

(A)

1. Wear gloves, laboratory coat, and safety glasses when handling specimens and reagents.

2. Prepare necessary supplies (kit, plate shaker, pipette, U-shaped microtiter plate).

3. Bring kit and all necessary reagents to room temperature before use.

4. Label worksheet and microtiter plate with sample IDs. Divide plate so that each specimen will occupy a set of 4 consecutive wells. The positive control will occupy the entire first row (8 wells). The negative control will occupy 4 wells.

5. Add 100 µL of TPPA sample diluent to the first well of each specimen and control set. Add 25 µL of TPPA sample diluent to the remaining wells in the plate.

6. Invert serum sample to mix. Add 25 µL of sample to the first well in each specimen set. Mix by pipetting up and down 5-6 times. Transfer 25 µL to the second well and mix 5-6 times. Repeat through well #4, discarding the 25 µL from well #4 for each specimen.

7. Reconstitute unsensitized and sensitized particle reagents. Gently mix to ensure thorough resuspension.

8. Add 1 drop (25 µL) of unsensitized particles (gray cap) to the 3rd well for each sample and control.

9. Add 1 drop (25 µL) of sensitized particles (red cap) to the 4th well for each sample and to wells 5-8 of the positive control.

10. Cover with a plate cover and shake for 30 seconds.

11. Incubate for 2 hours at room temperature.

12. Read, interpret, and record results on appropriate forms.

Figure 10.8
Procedures of performing (A) and interpreting results (B) of the *T. pallidum* passive particle agglutination assay (TPPA)
Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

remove any unbound conjugate, a chromogenic substrate for peroxidase is added. The resulting colour change is measured spectrophotometrically after adding a stop solution. The colour intensity is proportional to the amount of antibody present in the patient's serum. As a result of this configuration, the specificity and sensitivity of the test are superior to those of the first-generation treponemal ELISAs.

CIAs to detect treponemal antibody are almost exclusively used in large clinical laboratories in industrialized countries where labour costs are high and a large throughput of specimens would be anticipated. These assays either use the principle of attachment of specific antibody to antigen-coated beads and subsequent detection of beads that have been tagged with phycoerythrin conjugated to goat anti-human IgG,

(B)
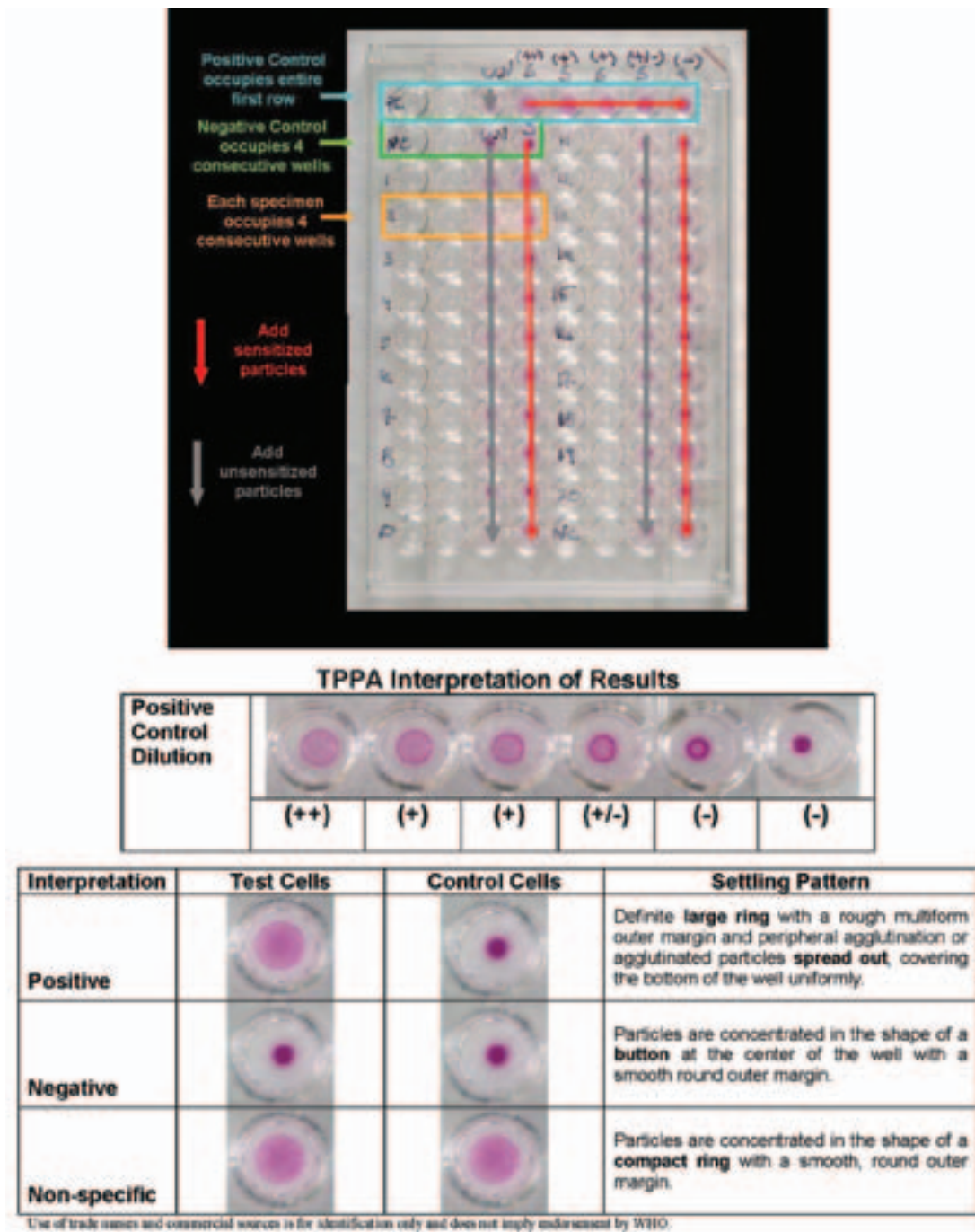
Figure 10.8 (*continued*)
Procedures of performing (A) and interpreting results (B) of the *T. pallidum* passive particle agglutination assay (TPPA)
Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

or detecting antibodies using an isoluminol-antigen conjugate to generate flashes of chemiluminescence that are detected by a sophisticated photomultiplier system (*16*).

Owing to the large number of EIAs and CIAs commercially available worldwide, a detailed description of the test procedure for each manufacturer's test is beyond the scope of this chapter. Therefore, the reader is requested to follow the instructions included in the package insert provided in each kit by the manufacturer.

10.3.2.4 Treponemal western blot (WB) assays

The treponemal WB test has been used as a confirmatory test for treponemal antibodies in the serum of patients with syphilis. Individual *T. pallidum* antigens are fractionated by polyacrylaminde electrophoresis of whole cell lysates. The resolved polypeptide bands of various molecular masses then are transferred to nitrocellulose membrane sheets. The sheets then are dried and cut into strips for use with individual serum samples. A patient's serum is diluted and incubated with each strip. If *T. pallidum*-specific IgG or IgM antibodies are present in the sample, they will bind to one or more of the 15-, 17-, 44.5-, and 47-kD molecular sized antigens on the strip. Any unbound antibody is removed by washing.

Bound antibodies are detected with alkaline phosphatase conjugated to anti-human IgG or IgM. The strip is then washed to remove the excess conjugate and finally reacted with a precipitating developing solution which forms purple coloured antigen bands. Reactivity at the specific band sites of the 15-, 17-, and 47-kD antigens is considered significant (Fig. 10.9). In contrast, reactivity at other positions on the strip is not considered significant. WBs have been used in the past to study the immune response to syphilis (*17*, *18*) and subsequently to select appropriate antigens to be included in line immunoassays.

10.3.2.5 Line immunoassays

Syphilis line immunoassays are designed to be confirmatory treponemal tests. They are intended for use as supplementary tests when routine treponemal test results are equivocal. Four recombinant proteins—TpN47, TpN17, TpN15, and TmpA—are coated as lines on strips of nitrocellulose membrane with a rigid plastic backing. The patient's serum is added to the test strips. If specific *T. pallidum* antibodies are present in the sample, they will bind to the individual antigen lines (*19*). After washing off any excess serum, a goat anti-human IgG or IgM, labelled with alkaline phosphatase is added, which will bind to any syphilis antigen–antibody complex formed. The subsequent addition of a substrate for the enzyme produces dark brown-coloured stripes, the density of which is proportional to the concentration of the specific antibodies present in the sample. When there are no *T. pallidum* antibodies present, only a low standard background colour may develop.

10.3.2.6 POC syphilis tests

There are many different manufacturers of POC diagnostic tests for syphilis worldwide. These tests are generally formatted either as lateral flow strip tests or as flow–through devices. In the lateral flow format, one or more recombinant antigens are striped onto a nitrocellulose immunochromatographic strip to capture specific treponemal antibodies, and a coloured
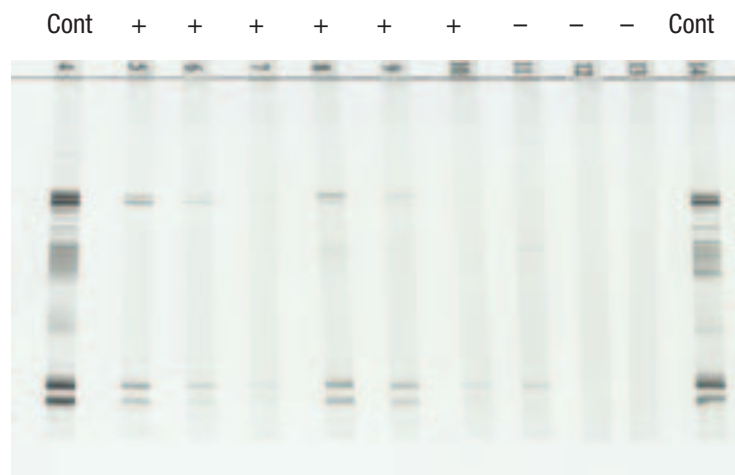

Figure 10.9
Detection of antibodies to *Treponema pallidum* by western blot (WB)
Source: Courtesy of Arnold Castro, Centers for Disease Control and Prevention, Atlanta, GA, USA.

line is produced upon binding either anti-human immunoglobulin linked to an enzyme, colloidal gold, or coloured latex particles.

A separate control line also is incorporated into the test, which either acts as a procedural control (in some tests) or indicates specimen adequacy (by indicating the presence of non-specific human immunoglobulin in the patient's specimen) (20–22). In the flow-through format, antigen spots replace lines and binding of specific antibody to the antigens occurs during passage through the membrane rather than when passing laterally along a membrane strip. All these tests provide a rapid indication of previous exposure to treponemal infection and have most conveniently been used to screen pregnant women in developing countries to prevent congenital syphilis. Use of these tests to screen for syphilis in pregnancy has been shown to be extremely cost effective (23) and has emerged as a key component of the World Health Organization Strategy for the Global Elimination of Congenital Syphilis (24). Use of these simple tests permits screening and same-day treatment at peripheral clinics that are remote from laboratories. These tests can be performed using whole blood (obtained by fingerprick), plasma, or serum; and do not require special equipment (including a refrigerator or centrifuge). Although the tests are easy to perform, strict adherence to the manufacturer's instructions is necessary to ensure ongoing accuracy of the tests. Adoption of POC tests at clinical sites requires ongoing supervision by a reference laboratory, including provision of an external quality assurance programme.

Apart from screening applications, these tests can be used to confirm the specificity of reactive non-treponemal tests in place of more complicated, laboratory-based treponemal assays. It is important to note that use of these rapid treponemal tests alone (i.e. without subsequent non-treponemal confirmation of reactive rapid tests), inevitably will result in considerable overtreatment, since, in common with all treponemal tests, they will probably remain reactive for life, even after provision of successful treatment. Ideally, a quantitative non-treponemal test should be performed to increase the specificity of a diagnosis of active infection, and to serve as a baseline to monitor treatment. For the same reason, these treponemal rapid tests cannot be used alone for serosurveillance of syphilis—unless a non-treponemal test is performed on all cases found to be reactive on rapid test screening and only those found to be dually reactive counted as cases.

Figure 10.10 shows an example of the procedure that can be followed and results that can be obtained in one example of a rapid, treponemal lateral flow device. For other devices, the manufacturer's instructions should be followed precisely. Both flow-through and lateral flow formats are amenable to multiplexing. As a result, dual treponemal/HIV immunochromatographic POC tests are currently in development.

It should be noted that there is currently at least one POC dual non-treponemal/treponemal test commercially available in some regions of the world (25, 26). By detecting both non-treponemal and treponemal antibody (Fig. 10.11), it is anticipated that these tests will greatly reduce the overtreatment rates inherent in current rapid testing and permit a single POC device to be used for syphilis serosurveillance.

10.3.3 Appropriate use of serological tests for syphilis

Table 10.1 shows the sensitivity and specificity of non-treponemal and treponemal syphilis tests for the different phases of the disease.

The conventional approach to serological testing for syphilis involves the screening of sera with a relatively inexpensive, sensitive, but relatively less specific and labour-intensive non-treponemal test and, if found to be reactive, confirmation with a more specific, but more expensive treponemal test. Throughout the years, this approach proved to be effective, particularly in those settings where the disease had been frequently encountered. The subsequent titration of confirmed-reactive sera using a quantitative non-treponemal test also provides a more precise interpretation of these results together with a baseline measurement (titre) against which the efficacy of therapy can be evaluated.

The recent development of treponemal ELISA and CIAs that can be automated has resulted in a change to this approach, particularly in many high-volume laboratories in industrialized countries where labour costs are high and the seroprevalence is low, such as in blood banks. Thus,
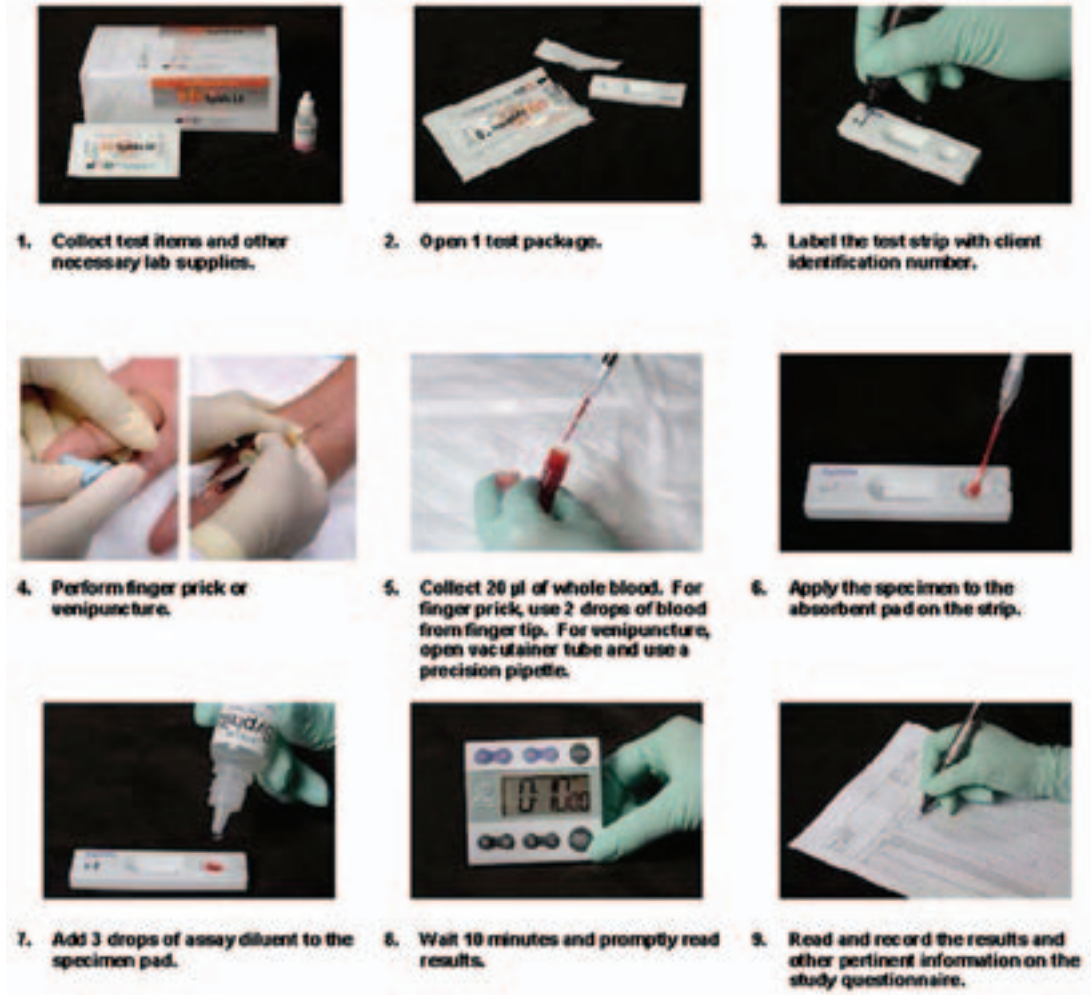
Figure 10.10

Procedures of performing (A) and interpreting results (B) of a rapid treponemal lateral flow device

Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

Table 10.1: Performance characteristics of selected serological tests for syphilis by stage of disease

Test	Sensitivity (%)				Specificity (%)
	Stage of syphilis infection				
	Primary	Secondary	Latent	Tertiary	
FTA-Abs	98 (93–100)	100	100	96	99
TPHA/PA	82 (69–90)	100	100	94	99
RPR	86 (81–100)	100	80 (53–100)	73 (36–96)	98
VDRL	80 (74–87)	100	80 (71–100)	71 (37–94)	98

FTA-Abs, fluorescent treponemal antibody absorption; RPR, rapid plasma reagin; TPHA, *T. pallidum* haemagglutination assay; TPPA, *T. pallidum* passive particle agglutination assay; VDRL, Veneral Disease Research Laboratory.

(B)
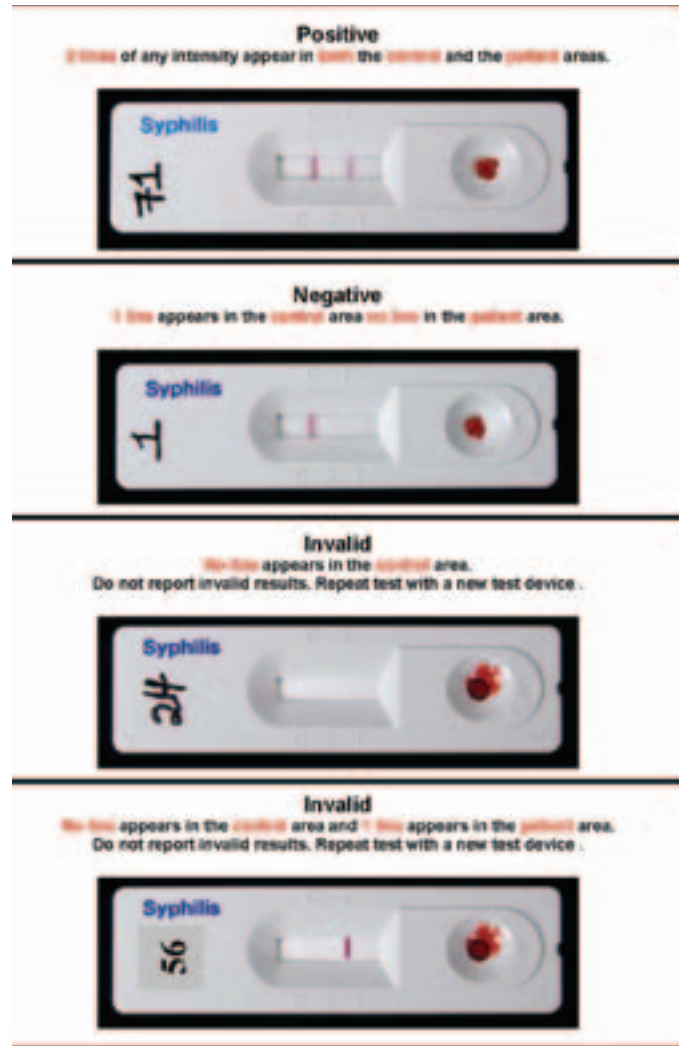
Figure 10.10 (*continued*)
Procedures of performing (A) and interpreting results (B) of a rapid treponemal lateral flow device
Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

batch screening of sera is performed using one of these newer ELISA or CIA treponemal tests, and only if a serum is found to be reactive, a reflex non-treponemal test is performed. If both treponemal and non-treponemal tests subsequently prove to be reactive, then a quantitative non-treponemal test should be performed as before.

These two approaches should theoretically produce the same result. However, the second approach results in the detection of treponemal test-positive, non-treponemal test-negative sera, which were not detected by the conventional approach (*27*). Since this pattern of serological reactivity can be found in very early syphilis, in previously treated early disease, and rarely in late

tertiary infection, considerable attention should be given to results of physical examination of the patient, previous history of disease, and recent sexual risk before initiating any treatment and partner notification activities.

Laboratories should consider the following factors before deciding the most appropriate (often cost-effective) approach to providing syphilis serological services for a particular population, namely: the number of sera screened for syphilis at a particular site; the syphilis seroprevalence (both non-treponemal and treponemal); the capital cost of instrumentation for ELISAs or CIAs; and the cost of non-treponemal and treponemal test kits and labour costs.
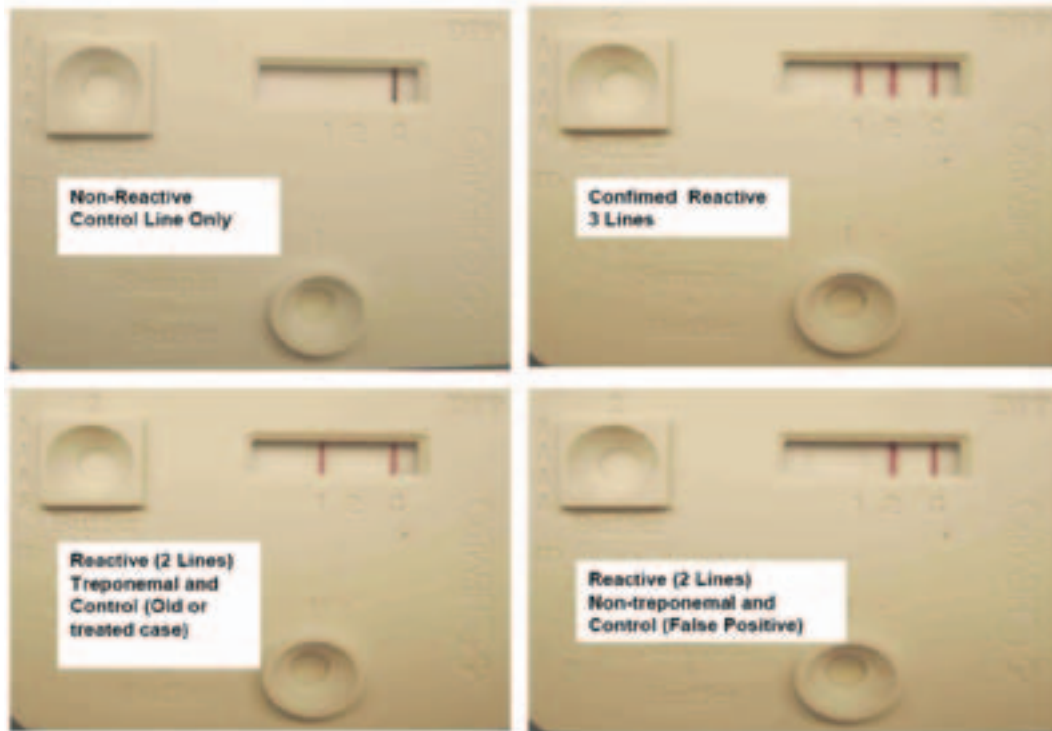
Figure 10.11
Point-of-care (POC), dual non-treponemal/treponemal test indicating patterns of reactivity and probable interpretation.

In cases where borderline reactions have been detected in any test, the test should be repeated, if possible, on a new serum specimen. If initial assays are non-reactive and primary syphilis is suspected, the tests have to be repeated after 2–3 weeks on a new serum sample. Finally, some patients given adequate treatment that was initiated very early in primary syphilis will remain antibody-negative.

Seroreversion of non-treponemal tests in patients who have been adequately treated usually occurs within a period of 6 months up to a few years, and is associated with the duration of infection and the antibody serum titre at the time of treatment (*28*). However, in some individuals, particularly those who were treated 1–2 years after initial infection, non-treponemal antibodies may persist for prolonged periods even after provision of effective therapy. These individuals are recognized as being "serofast". Seroreversion of treponemal tests also may occur in a minority of patients within a few years.

10.4 The laboratory diagnosis of congenital syphilis

Any skin and mucous membrane lesions present in a newborn birthed to a seropositive mother should be examined by dark-field microscopy, direct IF, or PCR for direct evidence of infection with *T. pallidum*. Saprophytic treponemes do not appear in the mouth until approximately 6 weeks after birth, so there is little chance of false-positive results being obtained from oral specimens.

The finding of a reactive serological test in a neonate may be the result of passive transfer of maternal antibody across the placenta during pregnancy and, therefore, cannot be considered diagnostic. However, the finding of a significantly higher RPR/VDRL titre (i.e. ≥4-fold higher) in the neonate's serum compared to the maternal titre or the detection of a significant rise in RPR/VDRL titre during a 3-month period have previously been considered indicators of congenital infection.

Modifications of the FTA-Abs test (FTA-IgM), specific EIAs, and line immunoassays that detect only IgM may be used to detect specific antitreponemal IgM, which is unable to cross the placental barrier. The finding of such IgM antibody in the baby's circulation is an indication of congenital infection, but specific IgM cannot be detected in all cases of congenital disease (29).

IgM-specific indirect IF tests have notoriously poor specificity and the FTA-IgM test, in common with its parent FTA-Abs test, is inherently subjective. In addition, the columns that were used to separate immunoglobulin fractions for use in the 19S-FTA-IgM test, which largely replaced the FTA-IgM test, are no longer available in many countries.

In IgM-specific EIAs, rabbit anti-human IgM (µ chain specific) antibody is coated onto the wells of microtitre plates. A measured dilution of the patient's serum is added to the wells of the plate. The rabbit anti-human IgM captures any available IgM antibody in the patient's serum. Purified *T. pallidum* antigen is added to the wells of the plate, and excess antigen washed away. A mixture of biotinylated human or rabbit anti-*T. pallidum* antiserum and streptavidin horseradish peroxidase is added to the wells of the plate. After rinsing off the unbound complexes, enzyme substrate and chromagen indicators are added. If the serum contains specific IgM antibodies to *T. pallidum*, a colour reaction takes place with the intensity of the colour proportional of the concentration of antibodies present.

10.5 Neurosyphilis

CSF abnormalities are common in patients with early syphilis who do not have neurological symptoms. While a high proportion of these have treponemes in their central nervous systems, they do not require the enhanced treatment required for neurosyphilis (30). As a result, routine lumbar puncture is not performed routinely in early syphilis unless clinically indicated. However, when indicated, CSF specimens should be examined for total protein and leukocyte counts and a VDRL-CSF test performed. The VDRL-CSF test has high specificity (99–100%) but low sensitivity for neurosyphilis (31, 32). Thus, while a reactive VDRL-CSF test is an indicator of neurosyphilis, a nonreactive test cannot be used as a means to exclude neurosyphilis. In contrast, the FTA-Abs CSF test has high sensitivity but low specificity for

neurosyphilis owing to passive transfer of specific IgG antibodies across the blood–brain barrier. Thus, when a negative FTA-Abs CSF test result is obtained, there is a high probability that neurosyphilis can be excluded (33).

10.6 Detection of anti-treponemal IgM in adult sera

As in other bacterial and viral infections, the synthesis of specific IgM antibodies is the first post-infection humoral immune response in syphilis. However, in syphilis, treponemal IgM antibody is not only present in patients with early syphilis but also may be found during the latent period and in patients with late disease. This phenomenon limits the value of IgM-specific assays in the diagnosis of adult disease.

10.7 References

1. Pillay A et al. Laboratory-confirmed case of yaws in a 10-year-old boy from the Republic of the Congo. *Journal of Clinical Microbiology*, 2011, 49(11):4013–4015.

2. Sparling PF et al. Clinical manifestations of syphilis. In: Holmes KK et al., eds. *Sexually transmitted diseases*, 4th ed. New York, McGraw-Hill Medical, 2008:661–684.

3. Larsen S et al. *A manual of tests for syphilis*, 9th ed. Washington, DC, American Public Health Association, 1998.

4. Wheeler HL, Agarwal S, Goh BT. Dark ground microscopy and treponemal serological tests in the diagnosis of early syphilis. *Sexually Transmitted Infections*, 2004, 80(5):411–414.

5. Hook EW III et al. Detection of *Treponema pallidum* in lesion exudate with a pathogen-specific monoclonal antibody. *Journal of Clinical Microbiology*, 1985, 22(2):241–244.

6. Chen CY et al. Diagnosis of gastric syphilis by direct immunofluorescence staining and real-time PCR testing. *Journal of Clinical Microbiology*, 2006, 44(9):3452–3456.

7. Gayet–Ageron A et al. Assessment of a real–time PCR test to diagnose syphilis from diverse biological samples. *Sexually Transmitted Infections*, 2009, 85(4):264–269.

8. Liu H et al. New tests for syphilis: rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene. *Journal of Clinical Microbiology*, 2001, 39(5):1941–1946.

9. Marfin AA et al. Amplification of the DNA polymerase I gene of *Treponema pallidum* from whole blood of

persons with syphilis. *Diagnostic Microbiology and Infectious Diseases*, 2001, 40(4):163–166.

10. Orle KA et al. Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 from genital ulcers. *Journal of Clinical Microbiology*, 1996, 34(1):49–54.

11. Larsen SA, Steiner BM, Rudolph AH. Laboratory diagnosis and interpretation of tests for syphilis. *Clinical Microbiology Reviews*, 1995, 8(1):1–21.

12. Ratnam S. The laboratory diagnosis of syphilis. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 2005, 16(1):45–51.

13. Nandwani R, Evans DT. Are you sure it's syphilis? A review of false positive serology. *International Journal of STD and AIDS*, 1995, 6(4):241–248.

14. Aktas G et al. Evaluation of the fluorescent treponemal antibody absorption test for detection of antibodies (immunoglobulins G and M) to *Treponema pallidum* in serologic diagnosis of syphilis. *International Journal of STD and AIDS*, 2007, 18(4):255–260.

15. Egglestone SI, Turner AJ. Serological diagnosis of syphilis. PHLS Syphilis Serology Working Group. *Communicable Diseases and Public Health*, 2000, 3(3):158–162.

16. Marangoni A et al. Evaluation of LIAISON Treponema screen, a novel recombinant antigen-based chemiluminescence immunoassay for laboratory diagnosis of syphilis. *Clinical and Diagnostic Laboratory Immunology*, 2005, 12(10):1231–1234.

17. Baker-Zander SA et al. Antigens of *Treponema pallidum* recognized by IgG and IgM antibodies during syphilis in humans. *Journal of Infectious Diseases*, 1985, 151(2):264–272.

18. Moskophidis M, Müller F. Molecular analysis of immunoglobulins M and G immune response to protein antigens of *Treponema pallidum* in human syphilis. *Infection and Immunity*, 1984, 43(1):127–132.

19. Lam TK et al. Comparative evaluation of the INNO-LIA syphilis score and the MarDx *Treponema pallidum* immunoglobulin G Marblot test assays for the serological diagnosis of syphilis. *International Journal of STD and AIDS*, 2010, 21(2):110–113.

20. Zarakolu P et al. Preliminary evaluation of an immunochromatographic strip test for specific *Treponema pallidum* antibodies. *Journal of Clinical Microbiology*, 2002, 40(8):3064–3065.

21. Diaz T et al. Evaluation of the Determine Rapid Syphilis TP assay using sera. *Clinical and Diagnostic Laboratory Immunology*, 2004, 11(1):98–101.

22. Peeling RW, Ye H. Diagnostic tools for preventing and managing maternal and congenital syphilis: an overview. *Bulletin of the World Health Organization*, 2004, 82(6):439–446.

23. Rydzak CE, Goldie SJ. Cost-effectiveness of rapid point-of-care prenatal syphilis screening in sub-Saharan Africa. *Sexually Transmitted Diseases*, 2008, 35(9):775–784.

24. Schmid GP et al. The need and plan for global elimination of congenital syphilis. *Sexually Transmitted Diseases*, 2007, 34(7):S5–S10.

25. Castro AR et al. Novel point-of-care test for simultaneous detection of nontreponemal and treponemal antibodies in patients with syphilis. *Journal of Clinical Microbiology*, 2010, 48(12):4615–4619.

26. Yin YP et al. A dual point-of-care test shows good performance in simultaneously detecting nontreponemal and treponemal antibodies in patients with syphilis: a multisite evaluation study in China. *Clinical Infectious Diseases*, 2013, 56(5):659–665.

27. Centers for Disease Control and Prevention. Syphilis testing algorithms using treponemal tests for initial screening—four laboratories, New York City, 2005–2006. *MMWR Morbidity and Mortality Weekly Report*, 2008, 57(32):872–875.

28. Romanowski B et al. Serologic response to treatment of infectious syphilis. *Annals of Internal Medicine*, 1991, 114(12):1005–1009.

29. Bromberg K, Rawstron S, Tannis G. Diagnosis of congenital syphilis by combining *Treponema pallidum*-specific IgM detection with immunofluorescent antigen detection for *T. pallidum*. *Journal of Infectious Diseases*, 1993, 168(1):238–242.

30. Rolfs RT et al. The Syphilis and HIV Study Group. A randomized trial of enhanced therapy for early syphilis in patients with and without human immunodeficiency virus infection. *New England Journal of Medicine*, 1997, 337(5):307–314.

31. Davis LE, Schmitt JW. Clinical significance of cerebrospinal fluid tests for neurosyphilis. *Annals of Neurology*, 1989, 25(1):50–55.

32. Castro R, Prieto ES, da Luz Martins Pereira F. Nontreponemal tests in the diagnosis of neurosyphilis: an evaluation of the Venereal Disease Research Laboratory (VDRL) and the Rapid Plasma Reagin (RPR) tests. *Journal of Clinical Laboratory Analysis*, 2008, 22(4):257–261.

33. Jaffe HW et al. Tests for treponemal antibody in CSF. *Archives of Internal Medicine*, 1978, 138(2):252–255.
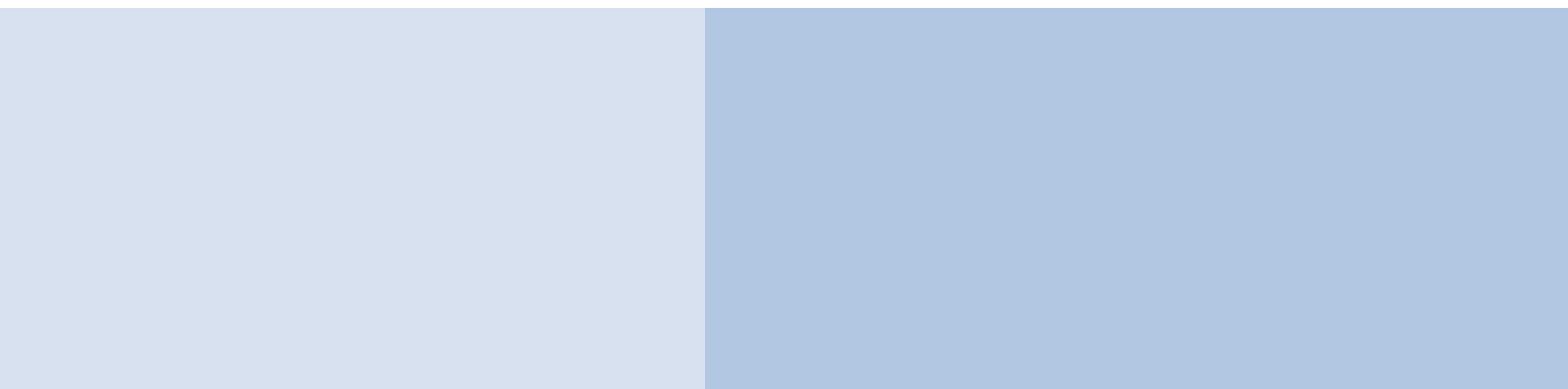
Chapter 11

Lymphogranuloma venereum (LGV)

11.1 Introduction

Lymphogranuloma venereum (LGV) is one of the three classical "tropical" sexually transmitted infections. It is caused by the distinct 'L' biovar of *Chlamydia trachomatis*, which contains serovars (L1, L2, L2a, L2b, L3) that are more invasive than those serovars responsible for the classical eye disease, trachoma (serovars A–C) and those causing non-gonococcal urethritis and associated infections of the genital tract (serovars D–K). LGV has worldwide distribution but is more prevalent in tropical and subtropical countries; for example, endemic in parts of East and West Africa, India and South-East Asia, South America, and the Caribbean. In most cases, these epidemiologic observations represent the classic clinical presentation of LGV, which is characterized by inguinal lymphadenopathy with or without an associated primary lesion (*1*). These symptoms are more commonly reported in men than women. In 2003, a cluster of rectal LGV infections, with characteristic lymph node involvement and/or proctitis or proctocolitis, was reported in men who have sex with men (MSM) in the Netherlands (*2*). Subsequent reports were made in many other countries in Europe, North America, Australia, and elsewhere, which previously only had sporadically imported LGV cases (*3–5*). The number of rectal *C. trachomatis* that belong to LGV or non-LGV biovar remains unknown due to a lack of systematic screening and widespread use of a discriminatory test.

Classically, LGV presents as a transient, herpetiform primary lesion of the external genitalia, but in many cases the lesion may pass unnoticed or manifest as an acute nongonococcal urethritis in men or be completely asymptomatic in women as a result of primary infection of the cervix. Most cases seek medical attention when the regional lymphatics become infected, as a result of lymphatic spread of the causative organism. In men, swelling of the inguinal and femoral glands often results in the formation of suppurating buboes on either or both sides of the inguinal ligament

(the "groove-sign", which may be seen also in a minority of chancroid patients) (*1*). In women, the perirectal and deep pelvic glands may become involved if the primary lesion is found on the cervix, and the patient may present with symptoms consistent with severe pelvic inflammatory disease (PID).

MSM may present with a severe ulcerative proctitis or proctocolitis with rectal pain, blood-stained discharge, markedly abnormal anoscopy, fever, and lymphadenopathy (*6*, *7*). In common with women, who may also present with such lesions, failure to treat the disease at this stage may result in the formation of perirectal abscesses, rectal strictures, fistulas, and chronic scarring. Apart from these complications, which arise as a result of acute inflammatory changes, chronic manifestations of the disease may result in blockage of the lymphatics draining of the genitalia or rectum, causing oedema. Severe lymphatic oedema is is termed "elephantiasis".

11.2 Laboratory tests

Until the early 1980s, isolation of *C. trachomatis* in cell culture remained the main method for diagnosis of chlamydial infection (see Chapter 5). Isolates from cases of LGV were recognized to grow more rapidly in tissue culture cells than non-L chlamydial isolates. Since the mid 1990s, nucleic acid amplification tests (NAATs) have become the tests of choice for diagnosis of chlamydial infections. These commercially available NAATs are substantially more sensitive than the older diagnostic tests (see Annex 3). However, these tests cannot discriminate between non-LGV and LGV strains. Subsequently, molecular assays have been developed that can differentiate between strains based on a deletion that occurs in the *pmpH* gene only in LGV isolates (*8–10*). Appropriate specimen collection, transportation, and storage are crucial for a high sensitivity and specificity of all diagnostic methods.

The specimens of choice for both culture and NAATs for LGV include swabs taken directly from primary lesions (when present), urethral swabs or first-catch urine specimens in men, endocervical swabs in women, and rectal swabs in MSM. Aspirates obtained from fluctuating regional lymph nodes rarely yield positive results. In contrast to other noninvasive chlamydial infections of the genital tract, LGV infection tends to elicit a significant antibody response. In the past, the chlamydial complement fixation test was extensively used for the diagnosis of the classical tropical infection with titres ≥1:64 being detected. Subsequently, the microimmunofluorescence test was used with broadly cross-reactive antibody titres ≥1:256 being detected. It should be noted that women with PID and others with complicated non-LGV may have similar antibody responses. The value of serology in the diagnosis of LGV proctitis and proctocolitis remains unknown.

11.3 References

1. Mabey D, Peeling RW. Lymphogranuloma venereum. *Sexually Transmitted Diseases*, 2002, 78(2):90–92.

2. Nieuwenhuis RF et al. Resurgence of lymphogranuloma venereum in Western Europe: an outbreak of *Chlamydia trachomatis* serovar L2 proctitis in The Netherlands among men who have sex with men. *Clinical Infectious Diseases*, 2004, 39(7):996–1003.

3. Rönn MM, Ward H. The association between lymphogranuloma venereum and HIV among men who have sex with men: systematic review and meta-analysis. *BMC Infectious Diseases*, 2011, 11:70–78.

4. Martin-Iguacel R et al. Lymphogranuloma venereum proctocolitis: a silent endemic disease in men who have sex with men in industrialised countries. *European Journal of Clinical Microbiology and Infectious Diseases*, 2010, 29(8):917–925.

5. White J, Ison C. Lymphogranuloma venereum: what does the clinician need to know? *Clinical Medicine*, 2008, 8(3):327–330.

6. Levine JS, Smith PD, Brugge WR. Chronic proctitis in male homosexuals due to lymphogranuloma venereum. *Gastroenterology*, 1980, 79(3):563–565.

7. Quinn TC et al. The polymicrobial origin of intestinal infections in homosexual men. *New England Journal of Medicine*, 1983, 309(1):576–582.

8. Morré SA et al. Real-time polymerase chain reaction to diagnose lymphogranuloma venereum. *Emerging Infectious Diseases*, 2005, 11(8):1311–1312.

9. Chen CY et al. The molecular diagnosis of lymphogranuloma venereum: evaluation of a real–time multiplex polymerase chain reaction test using rectal and urethral specimens. *Sexually Transmitted Diseases*, 2007, 34(7):451–455.

10. Alexander S, Martin IM, Ison C. A comparison of two methods for the diagnosis of lymphogranuloma venereum. *Journal of Medical Microbiology*, 2008, 57:962–965.

Chapter 12

Chancroid

12.1 Introduction

Chancroid is caused by *Haemophilus ducreyi* and the disease is transmitted exclusively by sexual contact, with direct invasion of the organism through healthy or abraded skin. It is approximately seven times more common in men than women, and transmission is linked to high numbers of sexual partners. Chancroid produces ulcers on the genitalia, typically the penile coronal sulcus in men and the vulva in women. Perianal chancroid may occur in receptive men who have sex with men and also among women who have engaged in penileanal sexual intercourse. Chancroid may be associated with suppurative inguinal lymphadenitis, particularly if there has been a delay in presentation to services or in making the correct diagnosis. Rarely, chancroid may be laboratory-acquired through accidental inoculation of *H. ducreyi* into fingers.

The incubation time is usually between 4–10 days. The genital ulcer starts as a tender papule that becomes pustular and ulcerates within 2 days. The ulcer is painful, irregular with undermined edges, and usually not indurated; these are the classical features that differentiate chancroid from syphilitic ulcers. However, it is important to note that the sensitivity of diagnosis based simply on the clinical appearance of the ulceration is poor. The base of the ulcer often is covered by a purulent and necrotic exudate, and bleeds easily when scraped or swabbed. Multiple lesions are common and they may merge to form very large ulcers. Unilateral painful inguinal adenitis also may occur, which, if not adequately and promptly treated, may lead to spontaneous rupture of suppurating lymph nodes (buboes). Atypical presentations of chancroid are common, and the disease can be easily confused with other genital ulcer diseases (GUDs), particularly genital herpes. Some genital ulcers may be infected with more than one GUD pathogen but this is less common nowadays as a result of the decline in relative prevalence of bacterial GUD.

The pathogen *H. ducreyi* is a short, non-motile, Gram-negative bacillus. It is a fastidious organism, and because of its complex nutritional requirements, highly enriched culture media are needed for its isolation. The growth of *H. ducreyi* isolates may be either aerobic or anaerobic. Growth is optimal at 32–33°C in a water-saturated atmosphere. Most strains, particularly on primary isolation, are dependent of carbon dioxide. *H. ducreyi* has few distinguishing biochemical characteristics: all strains reduce nitrate to nitrite, are positive for both oxidase and alkaline phosphatase, and require haemin (X factor) for growth.

Whilst chancroid was previously very common in certain parts of the world, the prevalence of the disease has waned dramatically since the 1990s, in part due to better access to antimicrobial agents, the roll-out of syndromic management, improved healthcare for sex workers, and sexual behavioural change in the era of HIV infection.

12.2 Overview of diagnostic procedures

The laboratory diagnosis of chancroid traditionally has been based on recovery of *H. ducreyi* in culture, which is a technically demanding procedure with low yield outside of highly skilled laboratories used to working with the pathogen (*1*). Although no commercial nucleic acid amplification tests (NAATs) exist for the diagnosis of chancroid, several in-house NAATs have been used to enhance diagnostic sensitivity (*1*). In addition, several research-based techniques have been described, including the use of monoclonal antibody-based antigen detection and DNA probes (*1*). Direct microscopy has very low sensitivity and specificity and, therefore, is of little use as a diagnostic tool for chancroid. The currently available research-based serological assays are only useful for sero-epidemiological purposes.

12.3 Specimen collection and transport

Specimens for *H. ducreyi* culture should be obtained from the base of the ulcer. Clean the ulcer with a dry gauze or a swab to remove crusts and superficial debris. Extensive cleaning is not required and may cause bleeding as well as being painful for the patient. Collect the exudate from

the base with a swab; the type of fibre used on the swab does not seem to affect culture sensitivity. Isolation of *H. ducreyi* from inguinal bubo pus has been much less successful than that from genital ulcer material and, therefore, is rarely performed. For optimal results, inoculate the specimens immediately on to the isolation media and keep in either a candle jar or a clinic-based incubator in a moist atmosphere at a temperature no higher than 35°C until final incubation. When culture media are not available at the clinical site, specimens may be transported at 4°C in a transport medium. A thioglycolate-haemin-based medium containing L-glutamine and bovine albumin seems to maintain the viability of *H. ducreyi* for several days at 4°C (*2*).

12.4 Isolation and identification of *H. ducreyi*

Bacteriological culture for *H. ducreyi* remains the main tool for diagnosis of chancroid in the clinical setting and for many years was the "gold standard" for evaluating other diagnostic methods. Successful culture is critically dependent on using freshly made media (ideally fewer than 7 days old) and attention to correct incubation conditions. However, with the advent of more sensitive research-based NAATs, it is now appreciated that culture may detect at best only 75% of *H. ducreyi* infections (*3*).

Initial attempts to cultivate *H. ducreyi* used fresh clotted rabbit blood heated to 55°C, fresh clotted human blood, and heat-inactivated human serum, but these methods were subject to recurrent problems of microbial contamination (*1*). This problem of contamination was subsequently addressed by adding vancomycin to semisolid chocolate agar at a concentration of 3 µg/ml (*4*). Using such selective media, Hammond et al. reported growth of *H. ducreyi* from 7 (44%) of 16 patients with clinically diagnosed chancroid (*4*). It should be noted that growth of some *H. ducreyi* strains may be inhibited at this concentration of vancomycin and such strains would require isolation on non-antibiotic containing culture media.

A number of selective artificial media have been developed and are reviewed in detail elsewhere (*5*). Nsanze et al. demonstrated that the yield of positive cultures may be increased when more than one type of culture medium was used to isolate *H. ducreyi* from genital ulcer material (*6*). Differences in nutritional requirements between *H. ducreyi* strains may account partially for these observations. A

medium containing GC agar base, 1–2% of haemoglobin, 5% fetal calf serum, 10% cofactors-vitamins-amino acids (CVA) enrichment and vancomycin (3 µg/ml) appears to have the highest sensitivity for the isolation of *H. ducreyi* from clinical specimens, with positive cultures being reported in up to approximately 80% of clinically defined chancroid cases (Fig. 12.1) (*5*). It has been noted, however, that some *H. ducreyi* isolates will not grow on this medium but may instead be isolated on a different medium made of Mueller–Hinton (MH) agar, 5% chocolatized horse blood, 1% CVA enrichment, and vancomycin (3 µg/ml) (*5*). To assist with optimizing *H. ducreyi* culture in clinical settings, two different media may be incorporated into a single biplate. More recently, a charcoal-based medium has been developed that avoids the need to add costly fetal calf serum, which may be a more cost-effective diagnostic tool in resource-poor settings (Fig. 12.2) (*7*). Annex 4 lists recipes for suitable media. In setting up facilities for *H. ducreyi* culture, it is advisable to use at least two of the above mentioned media and to define their sensitivities during pilot studies.

After inoculation, incubate the culture plates at 32–34°C in either a water-saturated atmosphere containing 5% carbon dioxide or, preferably, in microaerophilic conditions. Incubate the cultures for 48 hours prior to the initial reading and keep for 5 days before concluding they are negative. *H. ducreyi* colonies may vary in size depending on the time and temperature of incubation, the atmosphere, and the growth medium. Colonies are non-mucoid, raised and granular, have a greyish-yellow colour, and can be pushed intact in clumps across the surface of the agar with a bacteriological loop. Colonies are either translucent or opaque, and this variability may give the impression of a mixed, impure culture. Gram staining of smears from colonies shows Gram-negative coccobacilli in short chains, clumps, or whorls. Organisms are pleiomorphic in approximately 50% of the cultures. Individual bacteria may appear to have bipolar staining. In many areas of the world, almost all *H. ducreyi* isolates produce β-lactamase. This characteristic can contribute to a presumptive identification. For routine diagnostic work in endemic areas, there is no need for further identification. However, confirmatory identification may be needed for suspected isolates in non-endemic areas. A combination of some of the following methods can be performed to enable this process: oxidase test, nitrate reduction, porphyrin test, and detection of alkaline phosphatase.
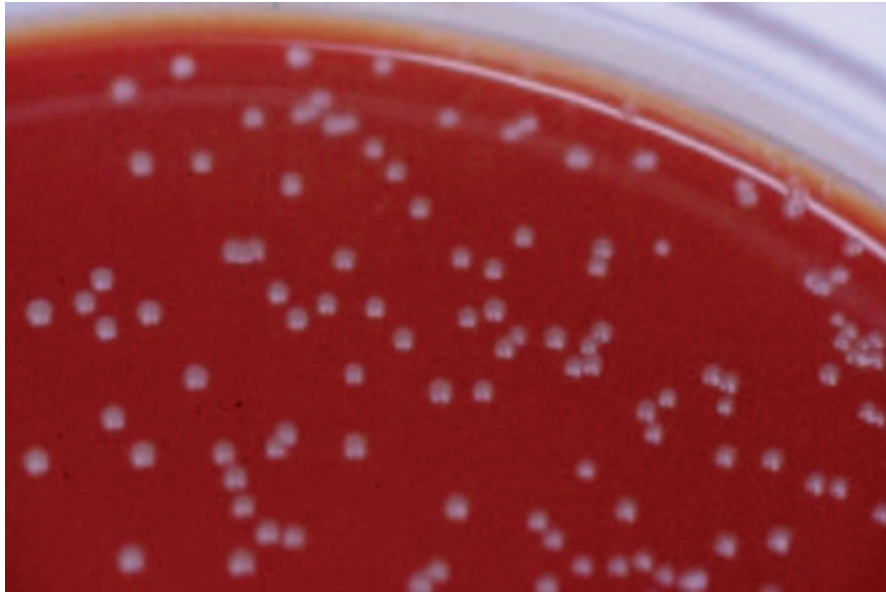
Figure 12.1
H. ducreyi growing on an enriched GC agar-based chocolate plate
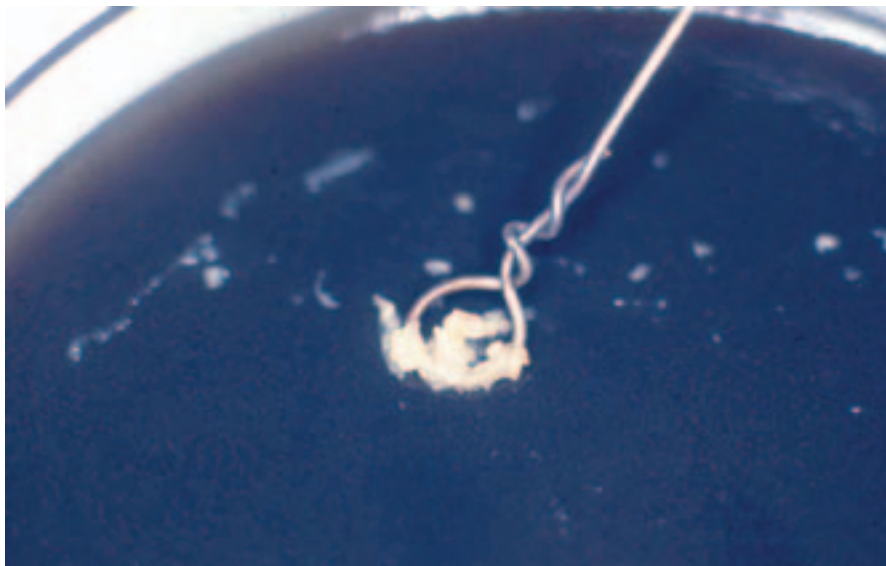
Figure 12.2
Clumps of *H. ducreyi* on a charcoal-based medium

- **Oxidase test**

  The production of cytochrome oxidase can be demonstrated by placing a few drops of tetramethyl-*p*-phenylenediamine hydrochloride on a strip of filter paper and by rubbing the growth from several colonies on to the impregnated area using a bacteriological loop. A colour change to blue to purple within 1 minute indicates a positive result.

- **Nitrate reduction**

  Prepare a dense bacterial suspension (McFarland standard 3, 10^9 CFU/ml) and transfer 0.04 ml to a small tube. Add 0.04 ml of 0.5 g/l sodium nitrate solution and 0.04 ml of 0.025 mol/l phosphate buffer, pH 6.8 and incubate in a water-bath at 37°C for 1 hour. Then add 0.06 ml of 8 g/l sulfanilic acid in 5 mol/l acetic acid and 0.06 ml of 5 g/l of α-naphthylamine in 5 mol/l acetic acid. The tube is shaken and, if a pink colour is observed, the test is positive.

- **Requirement for haemin (X factor)—the porphyrin test**

  The classical growth test with haemin-impregnated discs or strips cannot be used to detect *H. ducreyi.* The only reliable way of demonstrating haemin requirement is the porphyrin test. Make a dense bacterial suspension (McFarland standard 3, 10^9 CFU/ml) in 0.5 ml of a solution of 2 mmol/l δ-aminolevulinic acid hydrochloride in 0.1 mol/l phosphate buffer, pH 6.9 containing 0.8 mmol/l magnesium sulfate solution. Incubate in a water-bath at 37°C for 4 hours. Expose the substrate to Wood's light (wavelength 360 nm) in a dark room. A red fluorescence indicates the presence of porphyrins, i.e. no requirement for haemin. Thus, *H. ducreyi* should give a negative result and not exhibit a red fluorescence.

- **Detection of alkaline phosphatase**

  Make a dense bacterial suspension (McFarland standard 3, 10^9 CFU/ml) in a tube containing 0.5 ml of 0.3 g/l phenol-free disodium phosphate in 0.01 mol/l Sörensen's citrate-sodium hydroxide buffer, pH 5.6 and incubate the tube in a water-bath at 37°C for 4 hours. Add 4 drops of 5 g/l 2,6-dibromoquinone -4-chlorimide in methanol, shake, and stand the tube at room temperature for 15 minutes. Add 0.3 ml of *n*-butanol, shake, and stand for 5 minutes. A blue to purple colour in the butanol layer indicates a positive result.

- **Other characteristics of *H. ducreyi***

  Catalase, indole, and urease tests are negative. *H. ducreyi* is not considered to be saccharolytic. However, positive reactions for different carbohydrates have been reported. *H. ducreyi* possesses a wide range of aminopeptidase activity, and all tested isolates have shown activity with β-naphthylamide derivatives of L-lysine, L-arginine, L-alanine, L-glycine, glycyl-glycine, glycyl-L-alanine, and L-leucine.

12.5 Nucleic acid-based detection of *H. ducreyi*

At the time of writing (June 2012), no commercially available NAATs approved by the United States of America Food and Drug Administration exist for the detection of *H. ducreyi*. Several research-based NAATs have been described in the literature and utilize a number of different molecular targets, including the

H. ducreyi 16S rRNA gene, the *rrs* (16S)–*rrl* (23S) ribosomal intergenic spacer region, and the *groEL* gene (*1*). A robust multiplex polymerase chain reaction also has been developed for the detection of GUD pathogens, including *H. ducreyi,* and is in use in a number of international reference centres (*8*).

12.6 Nucleic acid probe technologies

DNA–DNA and DNA–RNA hydridization assays have been investigated as potential means to detect *H. ducreyi* in the laboratory, where they have proved to be 100% sensitive and 100% specific, but their role as a diagnostic tool to detect chancroid remains to be established (*1*).

12.7 Microscopy

Direct examination of clinical material on Gram-stained smears occasionally can be useful for the diagnosis of chancroid if typical small Gram-negative bacilli grouped in chains of "schools of fish", "railway tracks", or "thumb prints" are visualized. However, these classical morphological appearances are rarely seen in clinical practice. In addition, most genital ulcers harbour polymicrobial flora due to secondary contamination. The presence of Gram-negative bacilli on a smear, thus, can be misleading and contributes to the poor performance of microscopy as a diagnostic tool. Consequently, because of its low sensitivity and low specificity, Gram-staining of smears is not recommended for the diagnosis of chancroid.

12.8 Antigen detection of *H. ducreyi*

A number of monoclonal antibodies against prominent *H. ducreyi* antigens, including the 29 kDa outer membrane protein and lipo-oligosaccharide, have been utilized to detect *H. ducreyi* infection in several diagnostic formats, including immunofluorescence and immunolimulus assays (*1*). These assays are research-based and have not been used as a diagnostic tool in clinical practice.

12.9 Serology

Serological tests for the detection of *H. ducreyi* antibody currently are not commercially available. Human and rabbit serological responses to *H. ducreyi* infection have been detected by a number of technologies, for example, enzyme immunoassays, precipitation, agglutination, and

dot immunobinding (*1*). A humoral response to *H. ducreyi* infection develops during the ulcerative stage of chancroid but, based on clinical experience and human experimental inoculation studies, it appears that there is probably no acquired immunity to *H. ducreyi.* Serological tests offer little in terms of diagnostic assistance but would be a useful tool for those undertaking sero-epidemiological surveys for past infection with chancroid within communities.

12.10 Antimicrobial susceptibility testing

High-level plasmid-mediated resistance to sulfonamides, penicillins, kanamycin, streptomycin, tetracycline, chloramphenicol, and trimethoprim has been observed and described in *H. ducreyi* isolates. Plasmid-mediated chromosomal resistance patterns may vary greatly among geographically diverse areas. A large number of *H. ducreyi* isolates exhibit resistance to several antimicrobial agents. The continuing increase in drug resistance among sexually transmitted infection (STI) pathogens necessitates adequate surveillance of the susceptibility of clinical *H. ducreyi* isolates in those areas where chancroid still remains a significant clinical problem. However, as yet there are no standard procedures for antimicrobial susceptibility testing for this organism.

Most of the published studies have used the agar dilution technique to determine minimal inhibitory concentrations (MICs). One of the most suitable media is MH agar enriched with 1% haemoglobin, 5% fetal calf serum, and 1% IsoVitaleX™ enrichment supplement (Annex 4). Alternatively, MH agar can be replaced by GC agar base. The determination of antimicrobial MICs for *H. ducreyi* isolates is a cumbersome and technically delicate procedure and can be successfully performed only in specialized reference laboratories.

The list of antimicrobials to be tested should include those drugs locally recommended for treatment of chancroid as well as alternative therapeutic agents, those antimicrobials useful for the epidemiological study of *H. ducreyi* and, finally, newly developed drugs requiring microbiological assessment. Commonly tested antimicrobials include sulfamethoxazole and trimethoprim (used alone and in combination), tetracycline*,* chloramphenicol, erythromycin, kanamycin (or streptomycin), ciprofloxacin (or fleroxacin), and ceftriaxone (or cefotaxime).

The preparation of antimicrobial stock solutions and dilutions for use in MIC testing is described in detail in Chapter 4, section 4.8.4.2.

To prepare the medium, dissolve dehydrated MH agar and haemoglobin separately in distilled water. The volume of water used should be 16% less than the normal medium formula (to allow for the volume of the supplements and antimicrobial solution, which will be added later). Boil and dispense the MH and haemoglobin separately into containers in volumes appropriate for the number of plates to be prepared for each antimicrobial dilution, which will depend on the number of strains to be tested. Autoclave in tightly closed containers, allow to cool down to a temperature of 50–55°C in a water-bath, then mix together in one container and add 5% fetal calf serum, 1% *H. ducreyi* supplement, and 10% of antimicrobial solution. Mix gently and pour amounts of approximately 20 ml onto plates with an internal diameter of 9 cm. Once the agar has solidified, the plates may be stored for up to 1 week in sealed plastic bags at 4°C.

To prepare the inoculum, suspend the growth from a 24-hour subculture on enriched GC or MH agar (similar to isolation medium but without vancomycin) into tryptic soy broth (TSB) to a density of 10^8 colony-forming units (CFUs) per ml. *H. ducreyi* colonies often are so cohesive that a homogenous suspension cannot be obtained, even after vigorous shaking on a vortex mixer. The use of an orange 25G needle and syringe to break up the clumps, through repetitive drawing up and squirting out of the suspension, may assist with the creation of a more homogenous suspension as well as being less destructive to the bacilli. Centrifugation at low speed (500*g*) may be helpful to sediment large clumps. The density of the supernatant then is compared to McFarland 0.5 standard (10^8 CFU/ml). Dilute the suspension in TSB (1:10) to obtain 10^7 CFU/ml, and place 0.5 ml of this dilution into the corresponding well of a replicator seed block.

Warm the MIC test plates to room temperature and, if required, dry by placing in an incubator in an inverted position with the lids ajar. Transfer the prepared bacterial inocula to the test plates using a multipoint replicator, to produce spots containing approximately 10^4 CFU. Inoculate a control plate containing no antimicrobials first, followed by the plates containing the different

antimicrobials, starting with the lowest concentration for each agent. Finally, inoculate a second control plate. Allow the inocula to dry, invert the plates, and incubate at 33°C in a water-saturated atmosphere containing 5% carbon dioxide for 24 hours.

The MIC is the lowest concentration of antimicrobial that yields no growth, very few single colonies, or a fine, barely visible haze. The growth on both control plates should be confluent and without contamination. The determination of the MIC for sulphonamides is somewhat difficult since the end-points are less sharp than for other antimicrobials. A standardized reading and reproducible results are obtained if the second dilution on which there is a spectacular decrease of growth is taken as the MIC. It may be helpful to compare this growth to that on the control plates.

12.11 Conservation of isolates

To maintain the viability *of H. ducreyi* isolates in the laboratory, they should be subcultured every 4 days. Strains also can be preserved for up to 4 weeks by inoculating them on enriched chocolate agar stabs. For maintenance periods of several months, suspensions in skim milk can be stored frozen at –70°C. For long-term preservation, isolates can be suspended in a cryoprotective medium, such as fetal calf serum + 10% dimethylsulfoxide or skim milk + 20% glycerol, and stored in liquid nitrogen.

12.12 Medico-legal issues

Chancroid always should be viewed as an STI except in the rare instance of laboratory-acquired infection.

12.13 References

1. Lewis DA. Diagnostic tests for chancroid. *Sexually Transmitted Infections*, 2000, 76(2):137–141.

2. Dangor Y, Radebe F, Ballard RC. Transport media for *Haemophilus ducreyi*. *Sexually Transmitted Diseases*, 1993, 20(1):5–9.

3. Morse SA et al. Comparison of clinical diagnosis and standard laboratory and molecular methods for the diagnosis of genital ulcer disease in Lesotho: association with human immunodeficiency virus infection. *Journal of Infectious Diseases*, 1997, 175(3):583–589.

4. Hammond GW et al. Comparison of specimen collection and laboratory techniques for isolation of *Haemophilus ducreyi*. *Journal of Clinical Microbiology*, 1978, 7(1):39–43.

5. Morse SA. Chancroid and *Haemophilus ducreyi*. *Clinical Microbiology Reviews*, 1989, 2(2):137–157.

6. Nsanze H et al. Comparison of media for the primary isolation of *Haemophilus ducreyi*. *Sexually Transmitted Diseases*, 1984, 11(1):6–9.

7. Lockett AE et al. Serum-free media for isolation of *Haemophilus ducreyi*. *The Lancet*, 1991, 338(8762):326.

8. Orle KA et al. Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 from genital ulcers. *Journal of Clinical Microbiology*, 1996, 34(1):49–54.

Chapter 13

Donovanosis (granuloma inguinale)

13.1 Introduction

Donovanosis, also referred to as granuloma inguinale, is a chronic infection involving the skin, mucous membranes, and lymphatic system of the genitalia and perineal area (*1*). The occurrence of donovanosis is geographically limited, for example to Brazil, the Caribbean, India, Papua New Guinea, and southern Africa. The disease, which is of low infectivity, is transmitted between humans principally by sexual contact. The incubation time may be prolonged, varying between 1–12 weeks. The disease starts as an indurated subcutaneous nodule that erodes the skin surface to form a beefy red, hypertrophic, granulomatous ulcer with a well-defined border. The lesion bleeds easily on contact. The ulcer progresses slowly and may become painful when a secondary bacterial infection develops. Such secondary infection with other organisms may contribute to necrotic debris in the ulcer. New lesions may be formed by autoinoculation, and inguinal lymph nodes may become enlarged as a result of secondary infection (pseudobuboes). Donovanosis may spread haematogenously to bones, joints, and the liver; dissemination also may result in cutaneous lesions at extragenital body sites. Genital and perianal lesions at various stages may resemble lesions formed by other conditions, such as syphilis, chancroid, carcinoma, and amoebiasis.

Donovanosis is caused by *Klebsiella* (formerly *Calymmatobacterium*) *granulomatis*, a Gram-negative bacterium (1.5 × 0.7 μm), that can be observed enclosed in vacuoles in large histiocytic cells, where it is referred to as the "Donovan body" (*1, 2*).

13.2 Overview of laboratory diagnosis

Laboratory diagnosis depends on the visualization of Donovan bodies in stained smears obtained from clinical lesions or in stained histological sections of tissue biopsies. The organism can only be cultured with difficulty in specialist centres using monocyte/Hep-2

cell cultures; it is not yet possible to grow the organism on artificial media. In-house nucleic acid amplification assays have been reported in the literature but such assays are not available in most countries for routine diagnostic purposes (*3*).

13.3 Collection of specimens

Prior to taking the smear of the ulcer material, roll a cotton-tipped swab across the lesion gently to remove exudates due to secondary infection and/or debris in a manner that minimizes bleeding. A second swab should be rolled across the ulcer base, ensuring good sampling of the ulcer edges where Donovan bodies are most likely to be found, and then rolled evenly on a glass slide. Air dry the slide prior to transport to the laboratory. It should be noted that some practitioners prefer to use punch biopsy forceps to remove a small piece of tissue, which is then crushed, spread on a glass slide, and then air dried. A crush preparation facilitates the microscopic interpretation and enhances diagnostic value.

13.4 Microscopy

A simple rapid (1-minute) Giemsa method has been described (*4*). With this staining method, the slide is dipped five times in a fixative, six times in eosin solution, and six times in a thiazide dye mixture, and then rinsed with phosphate buffer, pH 6.8. Use of a 10% Giemsa or similarly diluted Leishman stain can be employed as an alternative following fixation of the material on the slide with methanol for 2–3 minutes. Cover the slide with diluted stain for 10 minutes (Leishman's stain) or up to 30 minutes (Giemsa stain) and then rinse the slide in a stream of buffered water or phosphate-buffered saline (pH 7.0–7.2). Following this, leave the slide to dry in air and then examine it with a light microscope using oil immersion (1000× magnification). The Donovan bodies appear as cocco-bacilli within large vacuoles (25–90 μm in diameter) in the cytoplasm of large histiocytes and occasionally in plasma cells and polymorphonuclear

leukocytes. The organisms are blue to purple in colour and often are surrounded by a prominent-clear to acidophilic pink capsule (Fig. 13.1). Typical bacteria resemble closed safety pins. Contamination with other bacteria often will be observed. Although microscopy of ulcer smears is the conventional way to diagnose donovanosis, Donovan bodies also have been identified from Papanicolaou (Pap) smears used in routine cervical cytology screening (5).

13.5 Histopathology

Histological examination of a biopsy may be helpful in the differential diagnosis between donovanosis and other conditions. An ulcer with a mixed inflammatory infiltrate of plasma cells, neutrophils, and histiocytes, with a conspicuous absence of lymphocytes, suggests donovanosis. Take a piece of tissue (3–5 mm thick) from the edge of the lesion with a punch biopsy forceps and place in a container with formaldehyde-saline fixative. The demonstration of Donovan bodies using Warthin–Starry silver impregnation reagent constitutes a diagnosis (6). Paraffinized biopsies should be cut in sections of 6 μm. After de-paraffination and hydration with distilled water, fix the sections on a glass slide with glycerol, dry, and treat with the acid silver nitrate solution at 43°C for 30 minutes. Wash with hot water, rinse with distilled water, dehydrate in 95% ethanol, and clear in xylene.

13.6 Culture

Following a report describing the culture of *K. granulomatis* from faeces in 1962, it subsequently proved very difficult to culture the organism from clinical specimens (1, 7). In 1997, two groups reported successful multiplication of *K. granulomatis* using different culture systems, i.e. a monocyte co-culture system and a modified chlamydial culture technique (8, 9). Apart from these isolated reports, there are no other in vitro culture techniques for the isolation of *K. granulomatis.* However, the organism can be cultured by inoculation of clinical specimens into the yolk sac of 5-day-old embryonated chicken eggs (10). The organism is detectable after 72 hours' incubation.
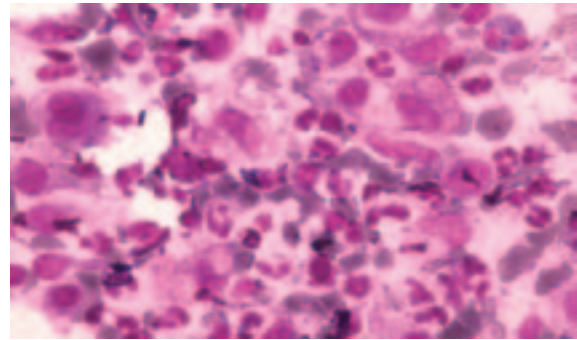
Figure 13.1
Giemsa-stained smear of ulcer material containing monocytes and Donovan bodies (1000×)

13.7 Nucleic acid amplification

A research-based diagnostic polymerase chain reaction (PCR) has been developed, which targets the *phoE* gene and incorporates post-amplification *Hae*III restriction enzyme-based digestion of amplicons (3). This method has been refined further into a colorimetric PCR test (11).

13.8 Serological tests

No serological assays are currently available to assist with diagnosis.

13.9 References

1. O'Farrell N. Donovanosis. *Sexually Transmitted Infections*, 2002, 78(6):452–457.

2. Carter JS et al. Phylogenetic evidence for reclassification of *Calymmatobacterium granulomatis* as *Klebsiella granulomatis* comb nov. *International Journal of Systematic Bacteriology*, 1999, 49(Pt4):1695–1700.

3. Carter JS et al. Diagnostic polymerase chain reaction for donovanosis. *Clinical Infectious Diseases*, 1999, 28(5):1168–1169.

4. O'Farrell N et al. A rapid stain for the diagnosis of granuloma inguinale. *Genitourinary Medicine*, 1990, 66(3):200–201.

5. de Boer AL, de Boer F, Van der Merwe JV. Cytologic identification of Donovan bodies in granuloma inguinale. *Acta Cytologica*, 1984, 28(2):126–128.

6. Freinkel AL. Histological aspects of sexually transmitted genital lesions. *Histopathology*, 1987, 11(8):819–831.

7. Goldberg J. Studies on granuloma inguinale. V. Isolation of a bacterium resembling *Donovania granulomatis* from the faeces of a patient with granuloma inguinale. *British Journal of Venereal Diseases*, 1962, 38(2):99–102.

8. Kharsany AB et al. Growth and cultural characteristics of *Calymmatobacterium granulomatis*—the aetiological agent of granuloma inguinale (donovanosis). *Journal of Medical Microbiology*, 1997, 46(7):579–585.

9. Carter J et al. Culture of the causative organism for donovanosis (*Calymmatobacterium granulomatis*) in HEp-2 cells. *Journal of Clinical Microbiology*, 1997, 35(11):2915–2917.

10. Anderson K, Demonbreun WA, Goodpasture EW. An etiologic consideration of *Donovania granulomatis* cultivated from granuloma inguinale (three cases) in embryonic yolk. *Journal of Experimental Medicine*, 1945, 81(1):25–39.

11. Carter JS, Kemp DJ. A colorimetric detection system for *Calymmatobacterium granulomatis*. *Sexually Transmitted Infections*, 2000, 76(2):134–136.
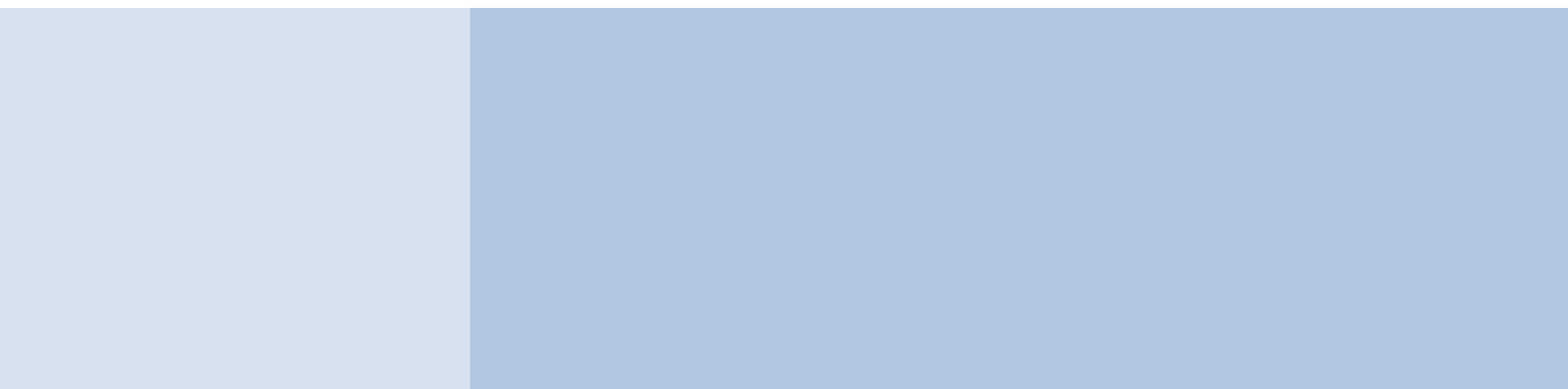
Chapter 14

Human papillomavirus (HPV) infections

14.1 Human papillomaviruses (HPVs)

HPVs are small (~55 nm diameter), icosahedral, non-enveloped viruses with a circular and supercoiled double-stranded DNA genome of approximately 8 kb (Fig. 14.1). These viruses typically infect the skin and mucosal surfaces of humans (*1*). Unlike most viruses infecting humans, papillomaviruses cannot be propagated by conventional in vitro culture; therefore, a classic antigenic and serotyping classification cannot be used for typing. Instead, a genotyping approach is used to identify and classify these viruses. Individual HPVs are referred to as types or genotypes, distinguished on their genomic sequence, and numbered in the order of the discovery. The L1 gene that encodes for the major component of the viral capsid (Fig. 14.2) is the region most conserved between individual types and used to form phylogenetic trees in taxonomy (Fig. 14.3) (*2*). Those HPVs with L1 sequence divergence of 2–10% are known as subtypes and less than 2%, variants. The term "genus" is used for the higher order clusters, named using the Greek alphabet, and within genus, small clusters are referred to as species and given a number. For example, the genus α papillomaviruses contain species α-9 and α-7, which contain, respectively, the

two most common causes of cervical cancer, HPV genotype 16 and 18 (*2*). There are more than 200 different types, of which approximately 100 are fully sequenced and 40 are known specifically to infect the anogenital mucosa of humans (mucosotrophic HPVs).

HPV types frequently detected in the anogenital tract are subdivided into low-risk (LR) and high-risk (HR) types, based on their relative risk for the rare complication of neoplasia (*3*). LR HPV types are typically found in low-grade intraepithelial lesions (non-precancerous lesions), as well as anogenital warts. HPV types 6 and 11 account for approximately 90% of genital warts (*4*, *5*). The HR HPVs are found in low- and high-grade lesions, as well as cancers of cervix and other anogenital sites (vulva, vagina, anus) (*6*). Collectively, HPV 16 and 18 are consistently responsible for approximately 70% of all cervical cancer cases worldwide (*7–11*). Apart from anogenital related cancers, oncogenic HPVs, type 16 especially, have a causal role in some oropharyngeal cancers, particularly those of the tonsil (*12*, *13*).
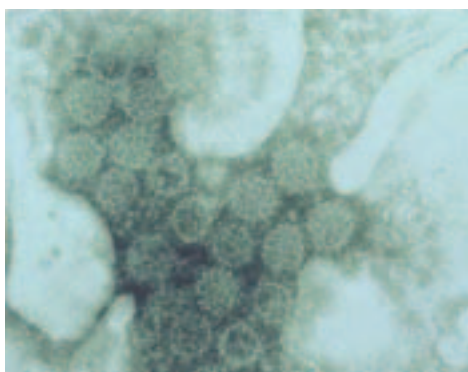

Figure 14.1
Electron microscopy of native HPV showing the virus particles in selected cells from a Papanicolaou (Pap) smear
Source: Courtesy of Colin Laverty, Sydney, NSW, Australia, 1978.
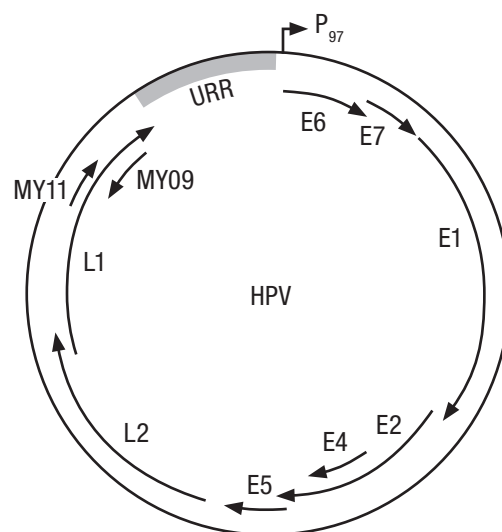
Figure 14.2
The genetic organization of HPVs (*48*)
URR, upstream regulatory region.
Source: Reprinted with permission from the World Health Organization (WHO).

Figure 14.3
Phylogenetic tree containing the L1 sequences of 118 papillomavirus types (*2*)
Source: Reprinted with permission from *Virology*.

14.2 Natural history of genital HPV infection

The majority of genital HPV infections are sexually acquired, transient, and cleared slowly by the host immune system (*14*). More uncommonly, in approximately 5–10% of cases, HPV infections may become persistent. Persistent HR HPV is a strong marker of risk for development of precancers, which in turn have the risk of progressing to cancer over many years if not treated (*15*, *16*). Established cofactors for the development of persistent infection and cervical cancer are cigarette smoking, long-term oral contraceptive use, high parity, early age at first delivery, and immunosuppressive states such as HIV coinfection (*17*). Whether there are specific host factors and/or other environmental factors that determine those destined to precancerous and neoplasia are yet to be determined (*18*).

Transmission of genital HPV largely occurs readily via direct sexual contact (genital skin with genital skin) with an infected individual, resulting in estimated lifelong HPV acquisition rates as high as 60–80% (*19*, *20*). The

median age of first sexual intercourse in many western countries, such as Australia, the United Kingdom, and the United States of America is 16 years old (*19*, *21*). Therefore, it follows that the highest rates of newly acquired HPV infections are observed in young women, peaking at an age soon after the onset of sexual activity, with estimates of up to 75% of HPV infections occurring in 15- to 24-year-old females. Recent data suggest that many new infections in young HPV-naive women are multiple infections (multiple HPV types detected) (*22*).

Less information is known about the natural history of HPV infection in males. Again, infection (penile, genital skin, and/or anal) is very common, occurring soon after onset of sexual debut, with rapid rates of acquisition as well as clearance (*23*, *24*). In addition, sexual behaviour with increased numbers of sexual partners increases the risk, whilst circumcision has a protective effect (*24*).

14.3 Immunology of HPV

The host immune response to HPV involves both humoral and cell-mediated compartments. Following natural

infections, the humoral antibody response is type-specific and first detected 6–18 months after infection (*14*). The response is weak and only approximately 50–60% of individuals who are HPV DNA-positive mount a measurable antibody response (*14*). Following natural infections, an individual may remain HPV DNA-positive despite development of specific antibodies; such individuals are not protected from developing subsequent disease from that HPV type. Clearance of HPV infection and resolution of clinical lesions such as genital warts is characterized by an effective cell-mediated immune response (*25*).

HPV serology is not used diagnostically. Sero-survelliance is useful to estimate on a population basis the age and prevalence of exposure in pre-vaccine settings. Post-vaccination serology is used as a measure of vaccine effectiveness. Protection corresponds to detectable neutralizing antibodies, although the actual mechanism of protection is not completely understood. Animal studies with host-specific papillomaviruses show neutralizing antibodies from primary infection that are protective, even following challenge with the respective virus (*26*). It is noteworthy that although durability of vaccine-induced antibodies in human trials is now documented beyond 8 years, an immune correlate of protection is yet to be defined (*27*).

14.4 Disease manifestations

Anogenital warts

Anogenital warts, also known as condylomata acuminata, are benign exophytic, papular or flat growths that may occur anywhere in the anogenital area. They are extremely common, particularly in young people commencing sexual activity, and largely diagnosed clinically. Lesions rarely may cause problems because of size and obstruction, but the biggest problems are cosmetic and psychosocial. Lesions have a tendency to recur after treatment. HPV types 6 and 11 cause the majority of anogenital warts (85–90%) (*5*).

Recurrent respiratory papillomatosis (RRP)

RRP is a very rare condition characterized by recurrent wart-like growths (papillomas) in the upper respiratory tract, although it can involve the lower respiratory tract,

resulting in significant morbidity and mortality. HPV types 6 and 11 largely cause these lesions. The larynx is most commonly affected, resulting in voice changes. In young children, airway obstruction may occur. The diagnosis is made clinically by observation of characteristic warty lesions at laryngoscopy/bronchoscopy. Lesions are benign, but recur frequently after treatment. There are two patterns of onset: the juvenile onset form (JORRP) in children (mean age 2 years) as well as the late onset form seen in adults (*28*).

Anogenital precancers and cancers

Persistent infection with oncogenic or HR HPV genotypes has been strongly associated with an increased risk of anogenital cancer. This has been best studied in the cervix, where criteria for cytological and histological identification of slowly progressing precancers were first defined. Nomenclature has changed throughout the years, and continues to evolve. Cancer precursors have been termed dysplasias (mild, moderate, severe) or cervical intraepithelial neoplasia (CIN1, 2, 3), and most recently high- or low-grade CIN1–3 lesions (HSILs, LSILs) (*29*). Terminology in other anatomic sites follows the same pattern: anal (AIN), vulvar (VIN), vaginal (VaIN), penile (PIN). In each case, precursor lesions (high-grade or grade 3) are asymptomatic, progress slowly, and diagnosed with histology. Screening is recommended only for cervical lesions, although approaches to anal screening are being evaluated (*30*). Currently treatment is only recommended for precursor (high-grade) lesions.

Strong laboratory and epidemiologic evidence links HPV to cervical cancer and persistent HR HPV is considered a necessary, but not sufficient causal factor for cervical cancer. In the absence of HPV infection, cervical cancer is extremely unlikely, and this tight link is the reason why vaccine aims to prevent the most frequent oncogenic HPV infections to prevent cancer, as well as the reason why HPV testing can assist in screening for cervical cancer. Current HPV vaccines target HR types HPV 16 and 18, which account for 70% of cervical cancer worldwide (*7–11*). The epidemiologic evidence is less established for other HPV-associated cancers, but HPV-attributable cancers include those in other anogenital sites as well as oropharynx (base of tongue and tonsil), resulting in a significant burden of disease (Table 14.1).

Table 14.1: HPV-attributable cancer in 2002

Site	Attributable to HPV (%)	Of which, HPV 16 and/or 18 positive (%)	Both sexes				
			Total cancers	Attributable to HPV	% all cancer	Attributable to HPV 16/18	% all cancer
Cervix	100	70	492 800	492 800	4.54	344 900	3.18
Penis	40	63	26 300	10 500	0.10	6 600	0.06
Vulva, vagina	40	80	40 000	16 000	0.15	12 800	0.12
Anus	90	92	30 400	27 300	0.25	25 100	0.23
Mouth	3	95	274 300	8 200	0.08	7 800	0.07
Oro-pharynx	12	89	52 100	6 200	0.06	5 500	0.05
All sites			10 862 500	561 100	5.17	402 900	3.71

Source: Adapted from Parkin DM. The global burden of infection-associated cancers in the year 2002. *International Journal of Cancer*, 2006, 118:3030–3044.

The proportion of cancers with HPV differs at each site, and HPV 16 is particularly prominent in non-cervical cancers.

14.5 Laboratory procedures for HPV detection

HPV testing relies on molecular methods. This is because, as noted earlier, conventional culture methods cannot culture HPV and serology is relatively insensitive. Testing methods for epidemiologic surveillance differ from those used in clinical applications. Epidemiologic methods require type-specific assays with high analytic sensitivity. Because clinical assays are using HPV as a marker of underlying disease, they are matched to disease endpoints. There are no treatments for HPV per se; treatments are directed to the HPV-associated cancer precursors or other HPV-related lesions. A wide variety of HPV assays are available commercially as well as developed by laboratories. Use in clinical settings requires either that laboratories rely on assays that have been validated by regulatory agencies for specific clinical indications, or that laboratories undertake validation of the clinical performance of the assay. Because cancer precursors are associated only with HR HPV, there are no clinical indications for LR HPV testing. There is some variation in which HR HPV types are clinically relevant, but most assays include (or cross-react) with the following 14 HR HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.

14.6 Clinical application of HPV detection assays

Clinical application of HPV detection assays includes the following:

- Primary screening either alone or in combination with cervical cytology (Fig. 14.4) in women ≥30 years of age. Longitudinal studies show HPV DNA testing has a higher sensitivity for predicting prevalent or later-to-develop high-grade dysplasia. Moreover, the negative predictive value (NPV) is extremely high and superior to cytology. Therefore, it is proposed that those who are negative for HPV DNA on two occasions can have an increased screening interval. This makes combination Papanicolaou (Pap) and HPV DNA testing cost effective (*31–36*).

- Triage of women with minimal cytology abnormalities (inconclusive, equivocal, or borderline) to discriminate those truly HPV-related and requiring follow-up (*37*).

- Post-ablative therapy for high-grade dysplasia and monitoring women for evidence of persistent/recurrent disease and as a test-of-cure (*32, 38*).

Use of HPV as a primary cervical cancer screen is being evaluated (*39*). Currently, while HPV testing is an excellent negative predictor of disease, it lacks specificity required for direct treatment. Cervical cytology is one option for a second test. Other molecular tests are being

Normal ASCUS/Borderline LSIL

HSIL Koilocytes
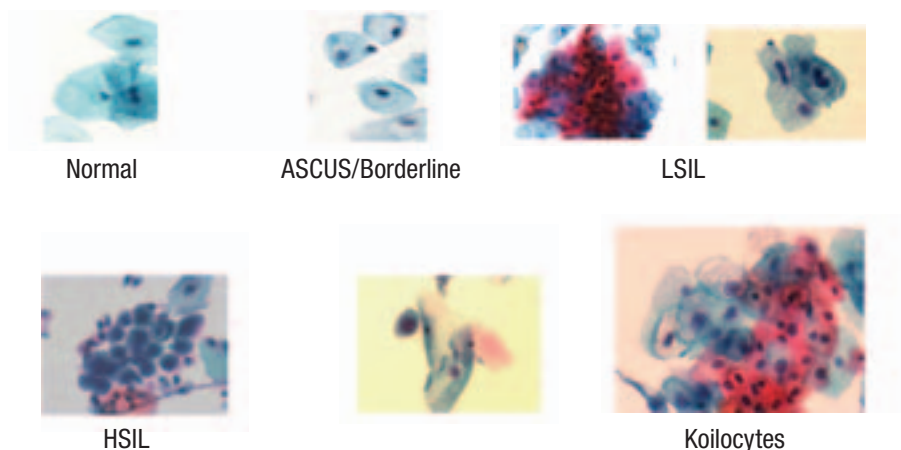
Figure 14.4
Papanicolaou (Pap) cytology of normal exfoliated cells, koilocytosis typical but not pathognomonic of HPV infection, atypical squamous cells of undetermined significance, LSIL, HSIL cellular changes.

evaluated, such as the host factor p16INK4a (p16), a cyclin-dependent kinase inhibitor. Elevated p16 indicates removal of the negative feedback control supplied by the retinoblastoma gene, pRB. When oncogenic HPV E7 proteins bind to pRB, p16 is overexpressed and elevated, representing active expression of HPV oncogenes (*40*) (Fig. 14.5). Moreover, in combination with increased expression of other host factors such as Ki67, overexpression of p16 correlates well with transforming viral infections (Fig. 14.6) (*41*).

14.7 HPV DNA detection methods

Historically, methods used to detect HPV DNA were direct-probe hybridization assays such as dot blot and Southern blot. These were labour-intensive and time-consuming, and also had low sensitivity, requiring large amounts of DNA in clinical samples. Clinical applications now rely on highly standardized assays that streamline sample handling and rely on some form of amplification for testing. Table 14.2 shows the current United States of America Food and Drug Administration (FDA)-approved tests.

The Hybrid Capture test (HC2), originated by Digene (Qiagen), was widely used in early studies and was the first FDA-approved assay. HC2 is a semiquantitative assay with a very good inter-laboratory comparison and high NPVs for CIN2/3 lesions (*42*). In addition, a low-cost version has been designed to be performed in low-resource settings requiring minimal equipment and training and operating as a rapid assay (*43*).
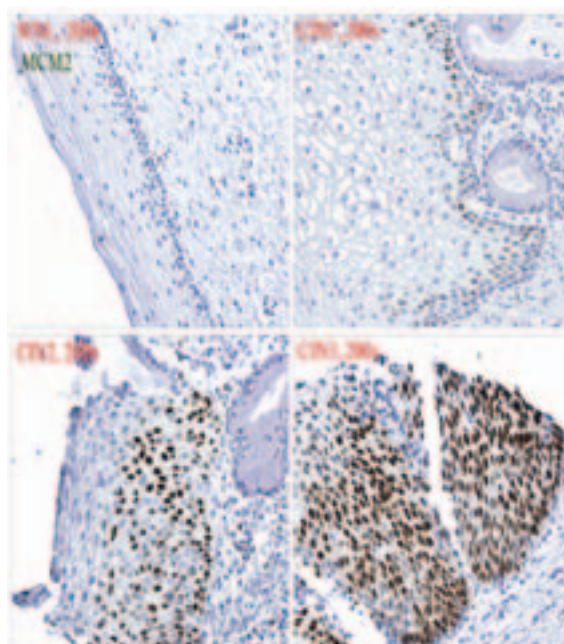
Figure 14.5
p16 not expressed in normal tissues, but being expressed progressively with increasing abnormality through CIN1 to CIN3.
Source: Reprinted with permission from Magnus von Knebel Doeberitz, Department of Applied Tumor Biology, Institute of Pathology, University of Heidelberg, Heidelberg, Germany.

Although for a long time only a research-based assay, this *care*HPV test is now available for use clinically. Its sensitivity for CIN2+ in a large trial in China was 90%, being not significantly different from HC2 with which it was compared; hence, promising as a primary screening
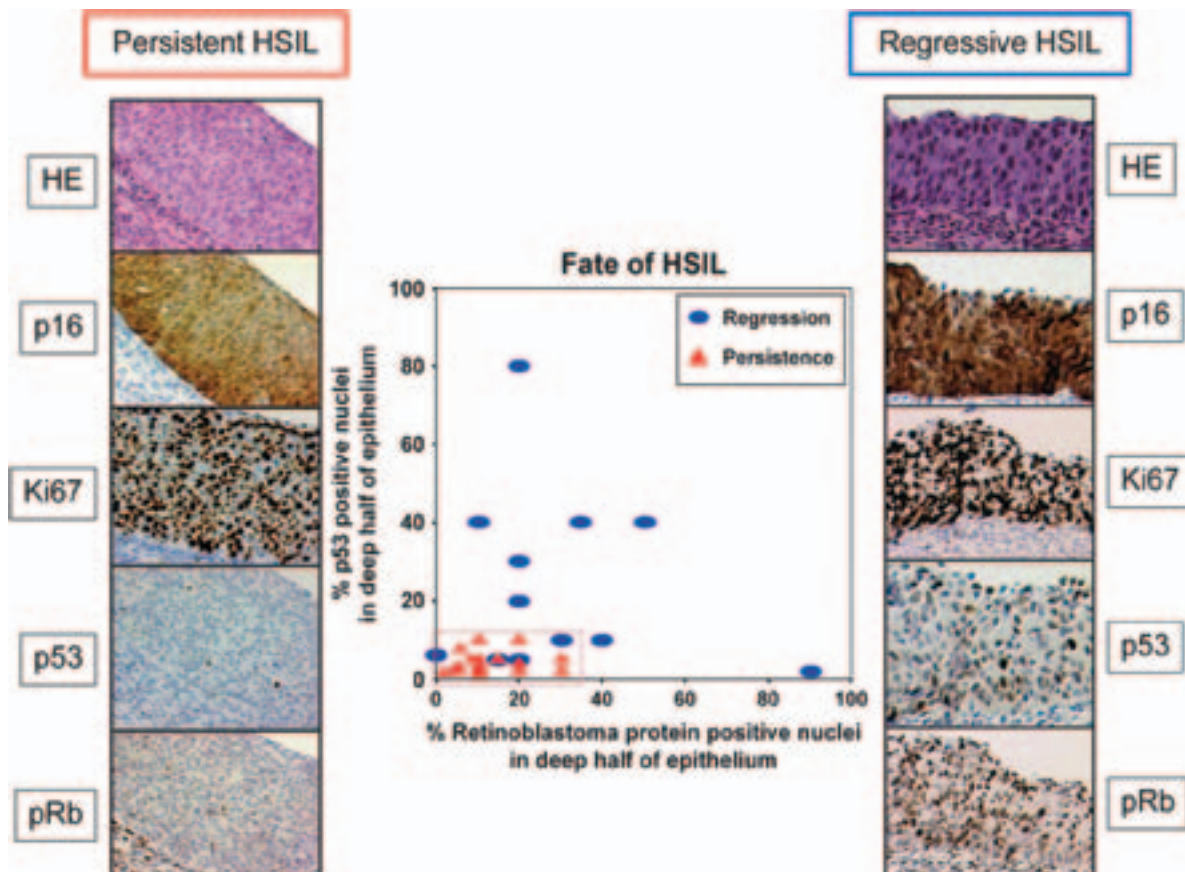
Figure 14.6
Various host and viral risk factor markers in HSIL. Center: Scatterplot of retinoblastoma protein (pRb) and p53 detection in the lower half of the epithelium of HSIL lesions that on follow-up persist (red triangles) or regress (blue open circles). Left: persistent HSIL; right: HSIL with regression (*41*).
Source: Reprinted with permission from the *American Journal of Surgical Pathology*.

Table 14.2: FDA-approved HPV tests

Test	Manufacturer	Method	Types (target)
Hybrid Capture (HC2)	Qiagen (Valencia, CA, USA)	Signal amplification	13 HR (genomic DNA)
Cervista HPV HR	Hologic (Bedford, MA, USA)	Probe amplification (Invader Technology)	14 HR (proprietary DNA)
Cervista HPV 16/18			HPV 16/18
APTIMA HPV	GenProbe (San Diego, CA, USA)	Target amplification (transcription-mediated amplification)	14 HR (E6/E7 RNA)
Cobas HPV	Roche (Pleasanton, CA, USA)	Target amplification (PCR)	14 HR (L1 DNA)

FDA, United States of America Food and Drug Administration; HPV, human papillomavirus; HR, high-risk types; PCR, polymerase chain reaction.

method for cervical-cancer prevention in low-resource regions (*43*). Results do not differentiate which of the HR types are present, but report results as indicating the presence of one or more types included in the assay.

Recently in an evaluation of various polymerase chain reaction (PCR) assays for primary screening and early detection of high-grade lesions, the sensitivity of target amplification assays for diagnosing underlying CIN2+

was reported as high, at approximately 96% (*34*, *44*). For example, the recently FDA-approved COBAS 4800 HPV test, which utilizes an automated sample preparation combined with real-time PCR technology to detect 14 HR HPVs in a single tube, compared favourably with HC2, with clinical sensitivity and specificity non-inferior to HC2 (*44*). Similarly, in the recently published final results of the POpulation-BAsed SCreening study AMsterdam (POBASCAM) trial—a population-based, randomized, controlled trial—an L1-based (GP5+/6+) PCR assay led to earlier detection of clinically relevant CIN2 or worse, lending support to the use of DNA testing for all women ages 29 years and older (*34*). The PCR-based assays largely targeting the L1 region are referred to as L1 consensus PCR assays. Different assays use different primers: the MY09/MY11, later superseded by the PGMY09/11 primers, result in a 450 bp amplicon (*45*). Additional described primer sets include the GP5+/6+, producing an amplicon of approximately 160 bp (*46*), while the SPF10 produce a short amplicon of 65 bp (*47*). The PGMY09/11 system is commercially available as the Linear Array HPV Genotyping Test (Roche), while the SPF system is known as the INNO-LiPA HPV Genotyping v2 (Innogenetics), and the GP5+/6+ systems as either RUO HPV Genotyping LQ Test (Qiagen) or the Multiplex HPV Genotyping Kit (Multimetrix, Heidelberg). For paraffin-embedded tissues, particularly archival ones, the assays detecting short amplicons such as the SPF primers are preferred, should the DNA be degraded.

For further steps in each of these assays, the reader should refer to the World Health Organization (WHO) HPV manual, which details each step along the way, including description of primers, appropriate controls, prevention of contamination, interpretation of data, etc. (*48*).

HPV mRNA *assays*

In addition to DNA assays, others detect mRNA expression of E6/E7 oncogenes (*49*, *50*).

Principles of these various molecular assays are described under the following headings (*51*):

Preanalytics

Suitable specimens and appropriate handling of clinical samples are essential in obtaining an accurate result. In the detection of HPV, suitable samples include swabs, scrapes, and tissue biopsies. Appropriate handling for swabs includes transporting dry or in a viral transport medium, whereas scrapes and biopsy should be collected in viral transport medium. Transportation is appropriate at room temperature within 24 hours or up to 4 days at 4°C. Assays with FDA approval specify collection and storage methods, and results would not be considered valid if collection and storage deviate.

Analytics

Following specimen collection, extraction or release of nucleic acid from samples must occur. Assays with FDA approval specify processing or extraction methods required for each assay. Results would not be considered valid if processing or extraction deviate. Use of commercial assays does not obviate the need for laboratories to maintain diligent quality assurance (QA) and quality control (QC) methods. Inclusion of positive and negative cell line controls can be useful in monitoring results. Processing water blanks through all steps of the assay are particularly crucial for monitoring false-positive results that may occur through cross-sample contamination.

Some assays include an endogenous host cell target, such as β-globin, to monitor the presence of amplifiable DNA. Samples negative for the endogenous target and HPV cannot be interpreted. Clinically approved assays include guidelines for monitoring and reporting assays. Well verified, in-house, laboratory-developed assays can also be used in routine diagnostics. Where an approved commercial assay is available, it is recommended by accrediting authorities that the latter should be utilized. Some of the advantages of doing so are that commercial assays usually include quality controlled reagents, as well as appropriate controls.

Laboratory quality assurance

Good microbiological practices should be followed, including appropriate positive and negative controls. Participation in proficiency panels and QA programmes is essential to determine assay performance and every effort must be made to participate in such programmes for each analyte.

Epidemiology/research testing

Numerous commercial and in-house assays are used in epidemiology and research. They vary in their performance characteristics as highlighted in studies of global proficiency in HPV typing (*52*). Table 14.3 shows examples of currently available commercial assays that target DNA or mRNA. Laboratories seeking to provide data for monitoring response to vaccination should be encouraged to follow guidelines in the WHO HPV Laboratory manual (see section 14.8 on HPV LabNet below).

14.8 Development of a WHO HPV LabNet

A WHO-convened group of experts met in Geneva in August 2005 and recommended the establishment of a global HPV LabNet, to contribute to improving the quality of laboratory services for effective surveillance and HPV vaccination impact monitoring, and to conduct training

Table 14.3: Various currently available HPV assays with name, target, and primer

HPV assay type	HPV region targeted (primer)
All assays	**L1/E1/E6/E7**
Linear Array (Roche)	L1 (PGMY)
PGMY-RBH	L1 (PGMY)
InnoLiPA (Innogenetics)	L1 (SPF10)
CLART (Genomica)	L1 (PGMY)
DNA chip (Biocore)	L1
Microarray (Genetel)	L1
DEIA LiPA assays	L1 (SPF10)
Microarray (Papillocheck)	E1
Type-specific PCR (GenoID)	L1
In-house PCR Luminex	L1 (PGMY-GP)
PCR Luminex (Multimetrix)	L1 (GP)
PCR EIA (GenoID)	L1
Abbott RealTime High Risk HPV	L1
Amplicor HPV Test	L1
APTIMA Gen-Probe (TMA)	E6/E7
NucliSENS EasyQ HPV	E6/E7

(*53–55*). It was envisaged that the HPV LabNet would speed up the introduction of HPV vaccines by facilitating the implementation of validated, standardized laboratory procedures; by developing QA system and proficiency testing; by training personnel; and by providing a network for surveillance. The WHO HPV LabNet was funded by a grant from the Gates Foundation, and was in operation under the auspices of WHO from 2006–2010. During these 4 years of operation, it focused on core activities of developing international standards for HPV DNA and serology assays, standardizing assays, and developing a laboratory manual and training programme emphasizing QA and QC. It collaborated with the National Institute for Biological Standards and Control (NIBSC) in developing the international standards for HPV types 16 and 18 DNA and HPV type 16 antibodies, which are available in the NIBSC catalogue. The WHO HPV laboratory manual was published in 2009 and is based on knowledge and experience gained through its international collaborative studies; it is available from WHO (*55*). The manual aims to assist in establishing the laboratory support required for implementation and monitoring of HPV vaccination programmes, and focuses on epidemiologic assays, not clinical assays. The HPV LabNet member laboratories continue to operate as a group to maintain a network of experts for optimal HPV testing, meeting annually at the international Papillomavirus Conference.

Progression markers

Not all women with persistent HR HPV, or for that matter all CIN3, progress to cervical cancer if untreated; therefore, other markers for predictors of progression are being investigated, such as p16. These progression markers hold promise together with HR HPV detection to more accurately diagnose high-grade dysplasia (*40*, *41*).

14.9 Conclusions

There has been an explosion of information on HPV and related diseases, including many new technologies for HPV detection. It is important to utilize appropriate tests whether it be for clinical applications or epidemiological purposes. Moreover, to ensure the best quality of results, laboratories performing such assays must adhere to good QA and QC practices.

Key Points:

- Oncogenic HPVs are the necessary cause of most cervical cancer and a proportion of other anogenital cancers, including some oropharngeal cancers. Genital HPVs are extremely common, with approximately 80% of the sexually active population being infected at some time of their life.

- Persistent oncogenic HPV infection is a prerequisite to precursor lesion development and subsequent neoplasia.

- Cancer is a rare outcome of this very common genital infection.

- HPV types 16 and 18 consistently cause 70% of cervical cancers worldwide.

- The precursor lesion to cervical cancer is cervical intraepithelial neoplasia grade 3 (CIN3); HPV types 16 and 18 cause ~50% of these.

- HPV detection relies on molecular technology as it is not readily cultivatable by traditional viral diagnostic methods and serology is not sensitive. Primary HPV screening is being incorporated into cervical cancer screening in various combinations with traditional cervical cytology (Pap smear test).

- The assays used clinically to screen for underlying CIN3 or cancer are different to those for use in epidemiological studies such as surveillance pre- and post-implementation of vaccination programmes. There are currently no clinical indications for LR HPV testing. Clinical tests require careful standardization of sample collection, processing, and testing, with correlation to clinical (not analytic) endpoints.

- It is important that there are high QA and QC for HPV detection. The WHO HPV LabNet, in collaboration with NIBSC, has developed international standards for both HPV types 16 and 18 DNA and HPV type 16 antibodies.

- For HPV detection assays, careful collection of clinical samples, transport to the laboratory, as well as handling within the laboratory is an imperative to prevent contamination and false-positive assays.

- HPV DNA detection is an excellent negative predictor (if HR HPV is not detected, disease is unlikely), but there are many false-positive results in predicting disease. Ongoing research is investigating the usefulness of certain progression markers to improve specificity for clinically important outcomes.

- With proper health programmes of HPV vaccination, the positive predictive value of cytology in detecting high-grade dysplasia will reduce and in the future, various HPV molecular detection methods are more likely to be utilized.

14.10 References

1. Stoler MH. Human papillomavirus biology and cervical neoplasia: implications for diagnostic criteria and testing. *Archives of Pathology and Laboratory Medicine*, 2003, 127(8):935–939.

2. de Villiers EM et al. Classification of papillomaviruses. *Virology*, 2004, 324(1):17–27.

3. Schiffman M et al. Classification of weakly carcinogenic human papillomavirus types: addressing the limits of epidemiology at the borderline. *Infectious Agents and Cancer*, 2009, 4:1–8.

4. Van Ranst M, Kaplan JB, Burk RD. Phylogenetic classification of human papillomaviruses: correlation with clinical manifestations. *Journal of General Virology*, 1992, 73(10):2653–2660.

5. Garland SM et al. Natural history of genital warts: analysis of the placebo arm of 2 randomized phase III trials of a quadrivalent human papillomavirus (types 6, 11, 16, and 18) vaccine. *Journal of Infectious Diseases*, 2009, 199(6):805–814.

6. Grulich AE et al. Cancers attributable to human papillomavirus infection. *Sexual Health*, 2010, 7(3):244–252.

7. Bosch FX et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *Journal of the National Cancer Institute*, 1995, 87(11):796–802.

8. Clifford GM et al. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *British Journal of Cancer*, 2003, 88(1):63–73.

9. Muñoz N et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *New England Journal of Medicine*, 2003, 348(6):518–527.

10. Wallboomers JM et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*, 1999, 189(1):12–19.

11. de Sanjose S et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncology*, 2010, 11(11):1048–1056.

12. D'Souza G et al. Case-control study of human papillomavirus and oropharyngeal cancer. *New England Journal of Medicine*, 2007, 356(19):1944–1956.

13. Hong AM et al. Squamous cell carcinoma of the oropharynx in Australian males induced by human papillomavirus vaccine targets. *Vaccine*, 2010, 28(19):3269–3272.

14. Carter JJ et al. Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection. *Journal of Infectious Diseases*, 2000, 181(6):1911–1919.

15. Wallin KL et al. Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer. *New England Journal of Medicine*, 1999, 341(22):1633–1638.

16. McCredie MR et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *Lancet Oncology*, 2008, 9(5):425–434.

17. Muñoz N et al. Chapter 1: HPV in the etiology of human cancer. *Vaccine*, 2006, 24(Suppl 3):S1–10.

18. Moore EE et al. The roles of genetic and environmental factors on risk of cervical cancer: a review of classical twin studies. *Twin Research and Human Genetics*, 2012, 15(1):79–86.

19. Dunne EF, Markowitz LE. Genital human papillomavirus infection. *Clinical Infectious Diseases*, 2006, 43(5):624–629.

20. Garland SM et al. Human papillomavirus prevalence among indigenous and non-indigenous Australian women prior to a national HPV vaccination program. *BMC Medicine*, 2011, 9:104.

21. Rissel CE et al. Sex in Australia: first experiences of vaginal intercourse and oral sex among a representative sample of adults. *Australian and New Zealand Journal of Public Health*, 2003, 27(2):131–137.

22. Nielsen A et al. Type-specific HPV infection and multiple HPV types: prevalence and risk factor profile in nearly 12,000 younger and older Danish women. *Sexually Transmitted Diseases*, 2008, 35(3):276–282.

23. Giuliano AR et al. The human papillomavirus infection in men study: human papillomavirus prevalence and type distribution among men residing in Brazil, Mexico, and the United States. *Cancer Epidemiology, Biomarkers and Prevention*, 2008, 17(8):2036–2043.

24. Giuliano AR et al. Incidence and clearance of genital human papillomavirus infection in men (HIM): a cohort study. *The Lancet*, 2011, 377(9769):932–940.

25. Stanley M. Immunobiology of HPV and HPV vaccines. *Gynecologic Oncology*, 2008, 109(2 Suppl):S15–21.

26. Shope RE. Immunization of rabbits to infectious papillomatosis. *Journal of Experimental Medicine*, 1937, 65(2):219–231.

27. Garland SM, Smith JS. Human papillomavirus vaccines: current status and future prospects. *Drugs*, 2010, 70(9):1079–1098.

28. Somers GR et al. Juvenile laryngeal papillomatosis in a pediatric population: a clinicopathologic study. *Pediatric Pathology and Laboratory Medicine*, 1997, 17(1):53–64.

29. Darragh TM et al. The lower anogenital squamous terminology standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *Archives of Pathology and Laboratory Medicine*, 2012, 136(10):1266–1297.

30. Machalek DA et al. Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. *Lancet Oncology*, 2012, 13(5):487–500.

31. Cuzick J et al. Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. *Vaccine*, 2008, 26(Suppl 10):K29–K41.

32. Garland SM, Tabrizi S. HPV DNA testing: potential clinical applications. *International Journal of Health Promotion and Education*, 2006, 44(3):107–112.

33. Kjaer SK et al. Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. *British Medical Journal*, 2002, 325(7364):572–575.

34. Rijkaart DC et al. Human papillomavirus testing for the detection of high-grade cervical intraepithelial neoplasia and cancer: final results of the POBASCAM randomised controlled trial. *Lancet Oncology*, 2012, 13(1):78–88.

35. Rijkaart DC et al. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *International Journal of Cancer*, 2012, 130(3):602–610.

36. Ronco G et al. Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial. *Lancet Oncology*, 2010, 11(3):249–257.

37. Solomon D et al. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: baseline results from a randomized trial. *Journal of the National Cancer Institute*, 2001, 93(4):293–299.

38. Moore EE et al. Clearance of human papillomavirus in women treated for cervical dysplasia. *Obstetrics and Gynecology*, 2011, 117(1):101–108.

39. Sankaranarayanan R et al. HPV screening for cervical cancer in rural India. *New England Journal of Medicine*, 2009, 360(14):1385–1394.

40. Klaes R et al. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *American Journal of Surgical Pathology*, 2002, 26(11):1389–1399.

41. Baak JP et al. Combined p53 and retinoblastoma protein detection identifies persistent and regressive cervical high-grade squamous intraepthelial lesions. *American Journal of Surgical Pathology*, 2005, 29(8):1062–1066.

42. Snijders PJ, van den Brule AJ, Meijer CJ. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. *Journal of Pathology*, 2003, 201(1):1–6.

43. Qiao YL et al. A new HPV-DNA test for cervical-cancer screening in developing regions: a cross-sectional study of clinical accuracy in rural China. *Lancet Oncology*, 2008, 9(10):929–936.

44. Heideman DA et al. Clinical validation of the Cobas 4800 HPV test for cervical screening purposes. *Journal of Clinical Microbiology*, 2011, 49(11):3983–3985.

45. Gravitt PE et al. Improved amplification of genital human papillomaviruses. *Journal of Clinical Microbiology*, 2000, 38(1):357–361.

46. de Roda Husman AM et al. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *Journal of General Virology*, 1995, 76(4):1057–1062.

47. Kleter B et al. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *American Journal of Pathology*, 1998, 153(6):1731–1739.

48. Unger ER, Dillner J, Zhou T, eds. *Human papillomavirus laboratory manual*, 1st ed. Geneva, World Health Organization, 2010.

49. Ratnam S et al. Aptima HPV E6/E7 mRNA test is as sensitive as Hybrid Capture 2 assay but more specific at detecting cervical precancer and cancer. *Journal of Clinical Microbiology*, 2011, 49(2):557–564.

50. Ratnam S et al. Clinical performance of the Pre Tect HPV-Proofer E6/E7 mRNA assay in comparison with that of the Hybrid Capture 2 test for identification of women at risk of cervical cancer. *Journal of Clinical Microbiology*, 2010, 48(8):2779–2785.

51. Garland SM, Tabrizi SN. Pathogens relevant in sexually transmitted infections. In: Kessler HH ed. *Molecular diagnostics of molecular diseases.* Berlin/New York, Walter De Gruyter, 2010: 177–184.

52. Eklund C et al. The 2010 global proficiency study of human papillomavirus genotyping in vaccinology. *Journal of Clinical Microbiology*, 2012, 50(7):2289–2298.

53. Pagliusi SR, Garland SM. International standard reagents for HPV detection. *Disease Markers*, 2007, 23(4):283–296.

54. Brotherton JM, Kaldor JM, Garland SM. Monitoring the control of human papillomavirus (HPV) infection and related diseases in Australia: towards a national HPV surveillance strategy. *Sexual Health*, 2010, 7(3):310–319.

55. *Report of a WHO technical workshop on the role of laboratory detection of human papillomavirus in global disease prevention and control.* Geneva, World Health Organization, 2006 (http://www.who.int/biologicals/areas/human_papillomavirus/HPV%20technical%20workshop%2015-17%20Aug%202005%2006%20845.pdf, accessed 5 April 2013).
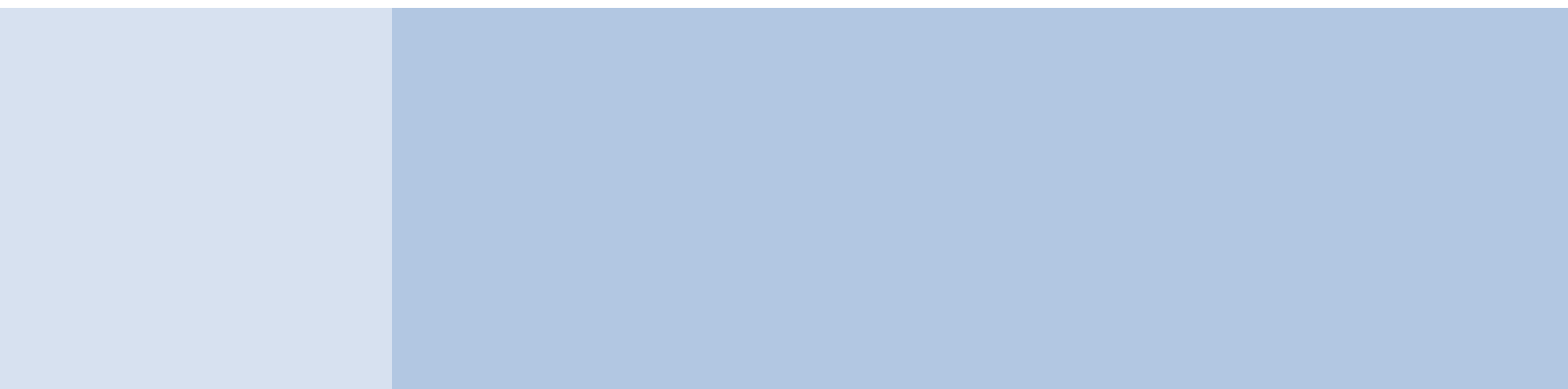
Chapter 15

Human immunodeficiency virus (HIV) infections

15.1 Introduction

The first cases of acquired immunodeficiency syndrome (AIDS) were described in 1981, and the causative virus was first isolated in 1983. Since then, the virus has spread worldwide, with an estimated 34 million people living with HIV worldwide and 2.7 million new infections in 2010 alone (*1*). Sub-Saharan Africa has been impacted the most, with 23.1 million people living with HIV and an estimated 1.9 million new infections in 2010. The prevalence of HIV has reached >30% among adults in some countries. Although effective antiretroviral treatments have been developed and treatment coverage has expanded to many resource-poor countries, for every new person put on treatment, there are estimated to be 2–3 new HIV-1 infections. Therefore, in the absence of an effective vaccine, accessible HIV diagnostics with counselling for prevention and referral of HIV-infected people to appropriate care are important strategies to slow down spread of the epidemic.

HIV is transmitted mainly through exchange of body fluids which can occur through the sexual route and via contaminated blood or blood products. HIV-infected pregnant women can transmit the virus to the infant during pregnancy, during delivery (perinatal transmission), or through breast-feeding (post-natal transmission). HIV transmission also is reported following organ transplantation when the donor is later found to be HIV-positive. To ensure safety, blood, blood products, and organ donors are now routinely tested for the presence of HIV or HIV antibodies to eliminate the possibility of HIV transmission.

HIV belongs to the family Retroviridae and subfamily Lentiviruses along with similar viruses such as visna virus, caprine arthritis-encephalitis virus, equine infectious anaemia virus, and simian immunodeficiency virus. The virion is approximately 100 nm in diameter with a conical capsid that contains 2 copies of genomic RNA (Fig. 15.1). The capsid protein, p24, is the major component of the virus and is further covered by a lipid envelope containing two glycoproteins, gp41 and gp120.

These surface glycoproteins are important for binding to CD4 T-cells, a first step to the infection process, as well as generating host immune responses to the virus. HIV is highly divergent, and several different subtypes or clades exist worldwide. Different subtypes are more prevalent in different parts of world. In addition, there are two major HIV types, HIV-1 and HIV-2. HIV-1 is subdivided into three groups: M (subtypes A–K), N and O. HIV-1 is the most common virus dominating the epidemic, with multitudes of subtypes and recombinant viruses. HIV-2 is mainly in West Africa, with occasional cases reported in many countries throughout the world. There are some differences among HIV-1 subtypes but for diagnostic purposes they cross-react heavily; therefore, antigens/proteins derived from a single subtype are adequate to diagnose infection irrespective of prevalent subtypes. As there is cross-reactivity among HIV-1 and HIV-2, additional specific antigens are required to accurately diagnose HIV-2 infection.

15.2 Diagnostic testing

15.2.1 Serological diagnosis of HIV infection

A serological diagnosis of HIV infection is obtained routinely by detection of HIV antibodies in the blood or other body fluids. Antibodies are elicited on average approximately 4–6 weeks after infection, although in some cases, development of detectable antibodies may take up to 3–6 months following infection. Therefore, HIV infection cannot be excluded on the basis of a negative test 4–6 weeks after documented exposure. During the initial period of virus replication, antibodies are absent and HIV diagnosis may not be made accurately using antibody-only tests. The acute window period can be shortened by using methods that can directly detect one or more components (p24 antigen or RNA) of HIV. Virus replication in the body elicits both humoral and cell-mediated immune responses, which reduces the virus level to a set point. The presence of detectable virus in

most untreated individuals continues to stimulate B-cell responses and antibody levels remain high throughout the subsequent period, unless the patient is highly immunocompromised, as in the later stages of the disease. Therefore, detection of HIV-specific antibodies is a very reliable marker for diagnosis of HIV infection (Fig. 15.2).

There are three main uses for HIV testing. HIV testing is performed to: ensure safety of blood, blood products, or organ transplant; conduct surveillance; and perform individual/patient diagnosis. Testing for blood safety usually is performed using the most sensitive and sophisticated testing procedures that detect both HIV antibody and p24 antigen or HIV-1 RNA to reduce the window period.

15.2.1.1 Enzyme immunoassays (EIAs)

Soon after discovery of HIV, EIAs were developed to diagnose HIV infection. These EIAs detected HIV-specific antibodies, a proxy for HIV infection, and used virus lysate as antigens. The first-generation assays were later replaced with second-generation assays that employ more specific antigens in the form of synthetic peptides or recombinant proteins. These assays were more sensitive and specific in detecting HIV antibodies; however, they still did not detect very early antibody responses in the form of IgM (immunoglobulin M), owing to their format and design. The third-generation EIAs utilize a sandwich format that includes antigens labelled with enzyme and are able to detect early IgM responses, thus reducing the window period. In the last few years, new fourth-generation EIAs were developed to reduce the window period further by combining detection of viral antigen (p24) in addition to HIV antibodies (Fig. 15.2). These combination antigen-antibody EIAs are very sensitive in detecting acute HIV infection prior to development of antibodies and are now used routinely for blood/blood products screening in many countries. Although molecular assays can detect viral nucleic acid a few days earlier than the p24 antigen detected by fourth-generation EIAs, the cost and complexity of nucleic acid detection may outweigh the benefits except in sophisticated blood banks.

Although EIAs are qualitative assays, a close review of optical density values may be informative. Those with high signal/cut-off ratios are very likely to be HIV-positive compared to those with low signal/cut-off ratios.
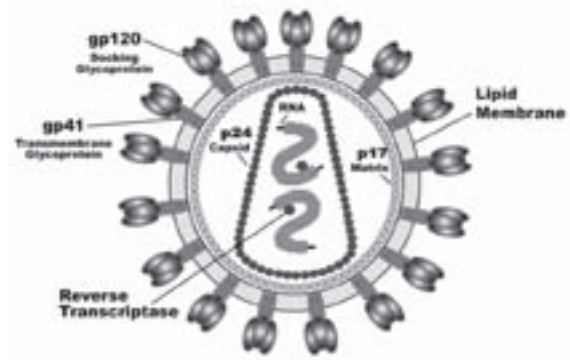
Figure 15.1
Structure and organization of HIV virus particle
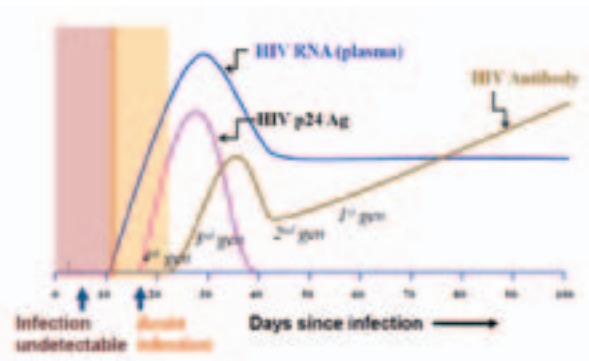
Figure 15.2
Early dynamics of virus replication and antibody development with markers of HIV infection. Approximate times for detection of antibodies by first-, second-, third-, and fourth-generation enzyme immunoassays are shown.

Therefore, the positive predictive value (PPV) of one or two reactive EIA results will be much higher if the results are interpreted in the context of signal. Those with a low signal, even when reactive by two EIAs, should be further tested with a more specific test or a follow-up sample to confirm infection.

15.2.1.2 Rapid tests

In the last few years, HIV diagnosis has moved increasingly from laboratory to non-laboratory settings as a result of availability of several HIV rapid tests. Worldwide, more than 100 million people were tested with HIV rapid tests in 2011 alone. HIV rapid tests come mainly in two different formats, which include immunoconcentration devices, and lateral flow cassettes or strips (Fig. 15.3). Because rapid tests are developed to detect HIV antibodies within a few minutes (1–15 min),

compared to EIAs, which may take up to 2–4 hours, the devices are optimized to accelerate antigen-antibody interaction. This requires the use of a high concentration of antigen and detection of antigen-antibody complexes with sensitive colour reagents, such as colloidal gold. Rapid tests are ideal for providing same-day results in a variety of situations such as testing hard-to-reach populations, home-based counselling and testing, provider-initiated testing, testing of pregnant women, and mobile testing. HIV rapid tests frequently are used in settings with low volumes of specimens to provide more cost-effective care. They can be performed using serum, plasma, or whole blood, facilitating the use of finger pricks specimens. Rapid tests are simple to perform and can be used outside laboratory environments by trained lay workers and counsellors, thus expanding access to HIV testing. The World Health Organization (WHO) and the United States of America Centers for Disease Control and Prevention (CDC) have developed an extensive training package that addresses various issues, including quality, accuracy, and safety (2). With expanded availability of care and treatment, there is an increased drive to provide counselling and testing to millions of people worldwide as an important component of prevention.

Owing to high demand and an expanded market, there are more than 50 test kits available worldwide, not all with desired performance characteristics. It is important to ensure that the rapid tests are manufactured to the highest quality according to good manufacturing practices and have performance characteristics equivalent to other diagnostic methods. WHO and CDC have qualification programmes to assess the quality of new rapid test kits (3, 4).

Recently, fourth-generation rapid tests capable of diagnosing acute HIV infection by detection of p24 antigen, in addition to detecting HIV antibodies, have been developed. Detection of p24 antigen can reduce the window period by a few days and identify people who are in the acute phase of infection. However, because detection of acute infection is a rare event even in high prevalence/incidence settings, a positive antigen test should be followed up by testing at 4 weeks or later to ensure seroconversion. A recent field evaluation of such a rapid test demonstrated that sensitivity and specificity

(A)   (B)
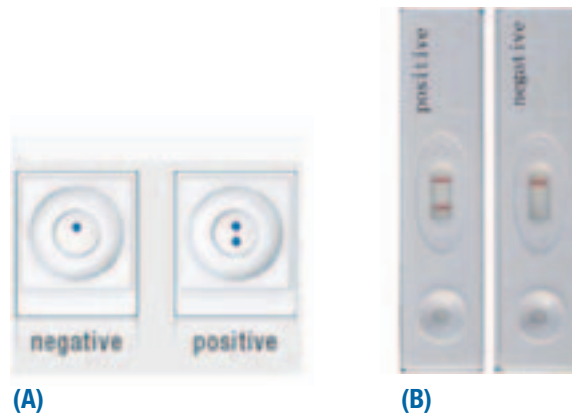
Figure 15.3
Example of HIV rapid tests, showing (A) flow-through immuno-concentration device, and (B) lateral flow device. Rapid tests with positive and negative results are shown.

of Ag detection are not yet acceptable (5). Moreover, since this technology is new and acute infections are rare, additional field validation that includes confirmation of all potential acute infections by nucleic acid testing would be important. As with any other testing, quality assurance (QA) measures are essential to ensure accuracy of rapid testing.

Several rapid tests have been developed that use oral fluid (OF) specimens collected using a swab. OF contains 1/500–1/1000 times less IgG than in blood specimens but sufficient to diagnose HIV infection. Most OF antibodies are transferred passively from the blood rather than locally elicited and contain a total complement of serum IgGs, albeit at a lower concentration. OF tests can simplify HIV testing further, making them more accessible while reducing biohazard risk associated with blood-based testing. Currently, at least three oral rapid test kits (OraQuick, ChemBio DPP, and Aware OMT) are commercially available.

15.2.1.3 Confirmatory assays

Although individual assays listed above are very sensitive and specific, false-positive and -negative results can occur. False-positive results can have major consequences at the individual level. Therefore, confirmatory assays were developed that can confirm initial positive results. These confirmatory assays, based on different formats and principles, include immunofluorescence assays (IFAs), western blot (WB), or

line immunoassays (LIAs). IFA involves use of slides with HIV-infected fixed cells. HIV antibodies, if present, are detected by fluorescein-tagged secondary antibodies and observed under a fluorescence microscope. IFA is no longer commonly used and has been replaced by other methods such as WB or LIA. WBs and LIAs involve the use of membrane strips with separated HIV- specific proteins or recombinant proteins or peptides. These strips are probed with serum specimens. The bound, HIV-specific antibodies are detected by secondary antibodies that are conjugated with an enzyme followed by a substrate that yields a coloured product. Figure 15.4 shows typical banding patterns on WB indicating presence of antibodies to specific viral proteins, including a positive control showing all the viral protein specific antibodies that can be detected with their characteristic pattern (Lane P). Individuals with new/recent infections have weak antibodies and to fewer proteins, typically p24 and gp120/gp160. As the infection progresses, antibodies develop to additional viral proteins and get stronger over time as shown for those with long-term infections. LIAs are similar to the WB assay but use selected purified recombinant proteins or peptides that are immunologically important for diagnosis. WBs and LIAs are expensive and require interpretation to establish a diagnosis based on a combination of antigen-specific antibodies (to p24 and

one or more envelope proteins) present in the blood. These assays detect only HIV-specific IgG. Therefore, they cannot be used to confirm presence of HIV-specific IgM or virus detected by more sensitive third- and fourth-generation EIAs, respectively. A testing algorithm that involves the use of two or more EIAs or rapid tests in a serial or parallel algorithm can provide results that are nearly as reliable as confirmatory WB or LIA assays but at a much reduced cost.

15.2.2 Detection of HIV RNA, DNA, or p24

HIV also can be diagnosed by direct detection of virus or virus components (p24 antigen, RNA, or proviral DNA). Detection of virus by culture or other methods is not commonly performed because of poor sensitivity compared to standard immunological or molecular methods and the complexity of virus culture techniques. Detection of p24 antigen or HIV-1 RNA or DNA play an important role when antibody-based diagnosis is not possible, such as in perinatally exposed infants or detecting acute infection in adults prior to development of HIV antibodies (6–10). Molecular detection of a nucleic acid target is more sensitive than p24 detection.

Depending on the stage of infection, HIV may be found primarily as pro-viral DNA in infected cells or as RNA in the blood (as a component of free virus particles and intracellular RNA). Commercial kits are available that can detect DNA and/or RNA either qualitatively or quantitatively. For diagnostic purposes, qualitative assays are sufficient and have applications for early detection of HIV infections in adults and infants. Acute infection detection by nucleic acid amplification tests (NAATs) has application in increasing safety of donor blood and blood products and possibly in high-risk populations. Many blood banks in developed and developing countries routinely use NAATs as part of their testing algorithms. Acutely infected individuals have high viral loads and are at high risk of transmission of infection to partners. Therefore, detection of acutely infected individuals has been promoted as part of overall HIV prevention strategy. However, detection of acute cases in most settings is low yield and very expensive considering the short window period (approximately 2 weeks).

Another important application for molecular tests is early diagnosis of HIV infection in perinatally exposed infants.
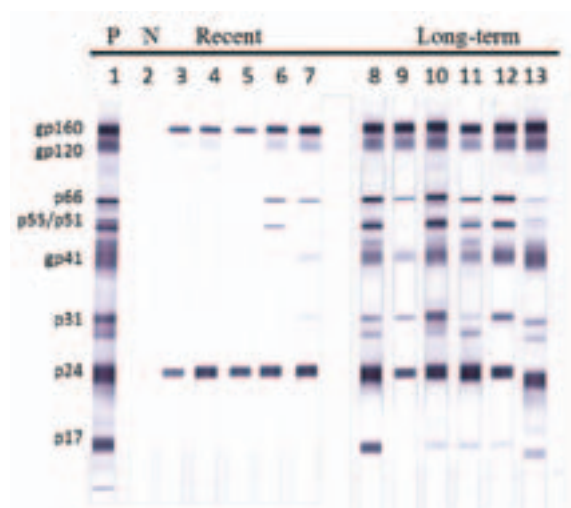

Figure 15.4
Confirmatory western blot assay and antibody banding pattern that may be observed during recent infections (strips 3–7) and long-term infections (strips 8–13). P, positive control; N, negative control specimens. Various virus-specific proteins are shown next to positive control.

Because all infants born to seropositive mothers have acquired HIV antibodies passively, routine antibody-based assays cannot be used to confirm or rule out HIV infection. Residual antibodies in uninfected infants persist and can be detected up to 18 months of age. Therefore, detecting the presence or absence of HIV RNA or DNA in infants 6 weeks or older is the most definitive way of diagnosing HIV infection in infants. For early infant diagnosis (EID) in resource-limited settings, the blood is spotted onto filter papers and dried (dried blood spot [DBS]) as a means to alleviate specimen collection, processing, transportation, and storage of blood specimens. With a major focus on prevention of mother-to-child transmission, molecular assays for early infant diagnosis have been implemented in a large number of laboratories. There are several commercial assays that are used for HIV diagnosis in infants. These assays include manual extraction of nucleic acids or state-of-the-art molecular platforms that offer advanced automation for the extraction of nucleic acids and qualitative detection of HIV-1 RNA and DNA. Manual nucleic acid extraction requires a significant number of steps and there is a potential for human error and polymerase chain reaction (PCR) contamination. With the need for higher quality, platforms have been designed to minimize user interventions and improve sample throughput. The qualitative detection of HIV-1 infection in infants has been reported to be sensitive enough to detect as low as 500 DNA copies/mL depending on the specimen source (plasma, whole blood, or DBS). Specimen pooling, which is often performed to detect acute infections in adults to increase efficiency and reduce cost, is not recommended for EID. Unfortunately, the HIV quantitative assays are not licensed in most countries for diagnosis but only for monitoring HIV infection. However, using a quantitative HIV RNA assay on plasma specimens prior to antiretroviral therapy (ART) initiation is a preferred confirmatory second test.

While performance of quality rapid serological testing for HIV at peripheral sites has become routine, point-of-care (POC) DNA PCR testing where laboratory infrastructure is minimal, is slowly advancing. Newer POC testing modalities that can be performed in resource-constrained settings are based on isothermal amplification with rapid detection of target sequences. Some POC assays utilize small portable instruments and

generally perform nucleic acid extraction and amplification, signal amplification, and detection. Field evaluation of these tests will be critical to validate their robustness and ability to provide accurate results.

15.2.3 Testing algorithms

For HIV diagnosis, two or more tests are combined into an algorithm to increase the PPV of an initial positive test result. In most situations, the most commonly used algorithm includes serial or sequential use of tests (serial algorithm). Although a negative result is usually given to a client based on a single test, a positive result is further confirmed with a second, different test. Based on the specificity of the tests and if the HIV prevalence is relatively high (>5%) in the population, two positive results are used to diagnose infection. In a low prevalence population, a third test is recommended before a positive HIV diagnosis is confirmed. The serial algorithm is logical and cost effective and includes more sensitive test as the first test followed by a more specific test to eliminate false-positive results. However, in some situations parallel algorithms are used that include using two tests simultaneously and results recorded as negative or positive based on concordant results. In the case of discrepant results, either a third test is performed or the client is tested at a later date to confirm or rule out recent seroconversion. Parallel algorithms may be more cost effective in a very high prevalence situation, high client volume, or when testing pregnant women in labour. It is important to select the correct combination of tests to provide an accurate diagnosis (11).

15.2.4 Dried blood spot (DBS) specimens

Although most diagnostic assays are developed for use with processed blood (serum or plasma specimens), specimen collection requires an experienced phlebotomist and equipment. Moreover, transportation of liquid specimens is cold-chain dependent, which is relatively expensive. DBS offers a simple and easy alternative as a sample for HIV serology and molecular testing. EIAs and WB have been optimized to work with DBS specimens (12). DBS specimens can be collected, stored, and transported at room temperature. A 6-mm punch normally is used to elute antibodies for testing. Depending on the assay used, some assay optimization usually is required to ensure optimal sensitivity and

specificity (*13*). With optimized assay systems, DBS is an ideal specimen for HIV surveillance.

As stated earlier, DBS is a convenient specimen that is routinely used for EID in many countries. Recently, assays have been optimized and validated for viral load monitoring with DBS specimens. Following effective treatment, the plasma viral load declines rapidly to below detection levels within a few weeks. However, intracellular RNA and DNA will persist and can be detected in DBS specimens. Therefore, the use of the DBS for longitudinal monitoring of patients on treatment requires caution.

15.3 Clinical laboratory monitoring of HIV infection

15.3.1 Role of CD4 testing in HIV clinical monitoring

Infection with HIV leads to development of AIDS, which is characterized by loss of the CD4 T-cells that are required for proper functioning of a person's immune system. CD4 testing is used in the clinical monitoring of HIV-infected people to determine the appropriate time to initiate ART, to monitor the efficacy of ART treatment, and to determine when to provide prophylaxis for opportunistic infections. WHO recommends ART for HIV-infected adults and adolescents with CD4 counts <350 cells/μl regardless of clinical symptoms (*14*). In resource-limited countries, the value for initiating therapy differs by country but usually ranges from 200 to 350 cells/μl. CD4 counts also are used as a tool to monitor disease progression and ART effectiveness. When a patient is not responding to treatment, CD4 data are used to determine change from first-line to second-line therapy, especially in countries where viral load testing is unavailable. The decision of when to start ART is critical in resource-limited

countries in the context of higher death rates and incidence of opportunistic infections (*15, 16*). When a CD4 count declines, HIV-positive individuals are more likely to become infected with opportunistic pathogens. ART is used when CD4 counts reach a certain cut-off to prevent the development of these infections. The normal CD4 count in children fewer than 5 years of age is higher than in adults and declines with age. This makes it difficult to access the eligibility of ART for children using absolute CD4 counts. CD4 percentages have been used to determine when to initiate ART in HIV-infected children.

15.3.1.1 Basics of the CD4 assay

In general, CD4 counts refer to the enumeration of CD4 T-cells, also called CD4-lymphocytes, or Helper T-cells. CD4 assays are used to determine the whole blood absolute CD4 cell concentration and/or the percentage of CD4 cells in the lymphocyte population. Normal CD4 counts are between 400–1600 cells/dl and the normal CD4 percentage of lymphocytes between 35–55% (*17*).

The standard methods for CD4 counts uses labelled monoclonal antibodies to identify blood cell surface molecules such as CD4, CD3, CD8, and CD45. The major white blood cells with labelled anti-CD4 and CD3 antibodies are depicted in Figure 15.5.

Blood cells that express the surface molecules of interest are identified with flow cytometers that detect the specific label of each of the monoclonal antibodies. Flow cytometers also differentiate blood cells by the cell's light-scatter properties. As an example of CD4 testing, a flow cytometric analysis with two-parameter dot plots is shown in Figure 15.6. The first dot plot identifies the white cell population by the light-scattering property of the cells and CD45 surface molecules. The box represented by R1 identifies the lymphocyte population
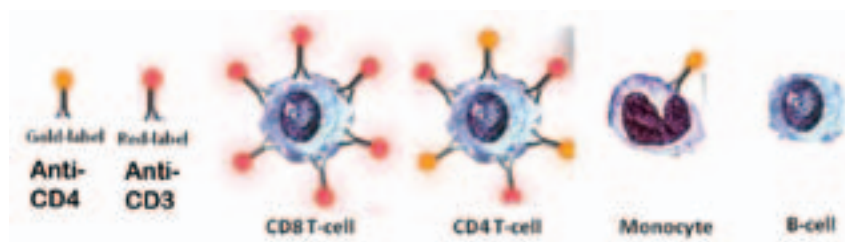

Figure 15.5
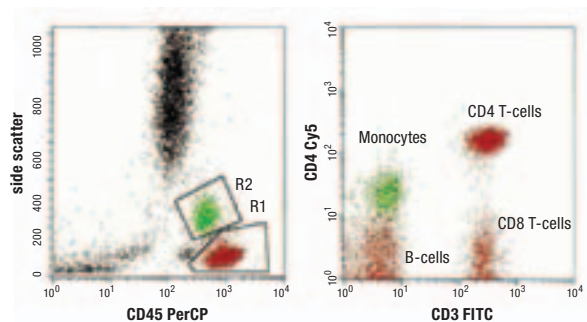**White blood cells with anti-CD4- and anti-CD3-labelled antibodies**

Figure 15.6
Example of flow cytometer dot plots from a CD4 assay

and R2 the monocyte population. The second dot plot only looks at the cells gated in R1 and R2 boxes from the first dot plot. The CD4 cells are the upper right cluster of cells having both CD4 and CD3 expression. CD8 T-cells are just below the CD4 cluster expressing CD3 but not CD4. B-cells are clustered in the lower left corner not expressing CD4 or CD3. Monocytes are the green cluster of cells above the B-cells, expressing low CD4 and not CD3. The flow cytometer can count the number of cells in each of the clusters to help determine the CD4 counts.

15.3.1.2 CD4 platforms and gating strategies

CD4 counts traditionally were determined using a dual-platform technique with a flow cytometer providing the percentage of CD4 cells in the white blood cell or lymphocyte population and a haematology analyser providing the absolute white blood cell or lymphocyte counts. Currently, single-platform methods are preferred and have been shown to improve the precision of the CD4 assay (15). The single-platform method determines the CD4 count on flow cytometers from a precise determined volume of whole blood sample. The single-platform methods are based either on a volumetric principle by counting CD4 cells in a unit volume of processed sample or based on comparison of the counts of a known number of microbeads added to the processed sample to the CD4 cells counts.

The recommended gating strategy for CD4 count identifies the lymphocyte population by CD45 markers and side-scattering properties. There are also a number of dedicated CD4 assays that use specific fixed gating strategies that are unique to the assay. Examples of

these assays include the FACSCount CD4 and Guava Auto CD4/CD4%.

15.3.1.3 CD4 assays

Table 15.1 shows the varieties of commercially available technologies that exist for CD4 testing. The table includes the manual methods for counting CD4 cells that rely on direct counting a microscope as well as the new POC CD4 assay, the Pima CD4. Prior to implementation of CD4 assays, consideration should be given to in-country or WHO policies on use of these CD4 tests in regards to: infrastructure and personnel needs for proper placement; supply and service logistics; and evaluation and monitoring of testing quality (16).

15.3.2 Quality assurance for CD4 testing

Development of QA guidelines for CD4 testing was driven by advances and experiences in both developed and resource-limited settings (17–19). It is important that specimens for CD4 testing be properly identified, collected in appropriate blood tubes, maintained at the ambient temperature but not exposed to extreme heat, and analysed within the timeframe recommended for the assay. Blood stabilizers, such as Cyto-chex and Transfix, have been used to extend the time in which a specimen can be analysed, particularly when specimen referral is required. Specimen should be processed following a written standardized procedure. Calibrated pipettes and properly stored reagents that are not outdated are important for reliable results. CD4 assays should be performed by trained and competent qualified laboratory personnel. All generated results, including dot blots, should be reviewed for accuracy, completeness, and correct identifiers. Equipment and instrument maintenance is necessary to be performed daily and at scheduled times. Service contracts on flow cytometers can extend the lifespan of the instrument and help ensure proper function of the instrument. Reagent inventory and a reliable supply chain are necessary to ensure no downtime in testing as a result of lack of reagents.

Quality control (QC) should be performed daily whenever CD4 testing is done. Stabilized blood can be used as QC material and is commercially available (19). Two levels of QC material, normal and low, should be tested as a

Table 15.1: CD4 instruments and assays

Type	Instruments/Company	Assay	Assay principle
High-throughput systems	Epic XL-MCL & FC500/ Beckman Coulter Brea, CA, USA	PLG CD4	Flow cytometry, bead-based
	FACSCalibur & Canto/ BD Bioscience San Jose, CA, USA	Tritest & MultiTest	Flow cytometry, bead-based
Mid- to low-throughput systems	FACSCount/BD Bioscience	FACSCount Reagent & CD4 Reagent	Flow cytometry, bead-based
	Guava PCA/ Millipore Billerica, MA, USA	Easy CD4, Easy CD4% Auto CD4/CD4%	Flow cytometry, volumetric
	CyFlow SL_3 & Counter/ Partec Gorlitz, Germany	Partec Easy CD4 & CD4%	Flow cytometry, volumetric
Manual systems	Beckman Coulter Invitrogen Oslo, Norway	Cyto-Spheres T4 Quant Kit	Direct observation of bead-rosetted cells Direct observation of immunocaptured cells
POC	Pima Analyzer/ Alere Technologies Jena, Germany	Pima CD4	Digital image of dual-labelled cells, volumetric

POC, point-of-care.

patient specimen. QC results should be reviewed daily. Alternatively, bead controls can be used to ensure the accuracy of the counting function of the CD4 instrument. Laboratories that perform CD4 testing should also be enrolled in an External QA/Assessment programme.

15.3.3 Viral load testing

Although CD4 is an important clinical parameter to understand the clinical status of patients and to initiate ART, viral load is the quantitative measure of amount of HIV in the blood and is an important parameter used to monitor efficacy of treatment over time. There are many commercially available viral load (VL) assays that are used primarily for managing HIV infection in conjunction with clinical presentation and other laboratory tests. The in vitro nucleic acid amplification technology is used to quantify HIV particles in human plasma. Reverse transcriptase (RT) PCR, nucleic acid sequence based amplification (NASBA) and branched chain DNA (bDNA) methods are used to quantify HIV RNA by PCR, isothermal nucleic acid amplification and signal amplification, respectively. Non-nucleic acid technologies are based on the detection of viral enzymes (RT) and proteins (p24 antigen) as a surrogate measure for HIV load. The VL assays differ in their sensitivity and dynamic range, and ability to detect and quantify different HIV subtypes. Notwithstanding the VL technology, human plasma is the standard specimen type, but the constraints of plasma processing, maintaining a cold chain, and storing specimens are genuine concerns. However, the use of DBS as a specimen source is promising, and primary care facilities can collect DBS and transport them to central laboratories for VL testing. Centralized testing currently is the norm in resource-

constrained countries, but POC testing can alter the landscape of where VL testing may be done routinely in the future.

15.3.4 Drug resistance testing

HIV drug resistance (HIVDR) refers to the ability of HIV to continue replicating in the presence of antiretroviral (ARV) drugs that usually suppress its replication. HIVDR is caused by mutations or changes in the relevant portions of viral RNA genome that are targeted by ARVs, which ultimately can lead to changes in enzymatic proteins essential for viral replication and enable HIV to replicate in the presence of the relevant ARVs. In resource-limited settings, the most common prescribed ARVs are against RT and protease (PR) regions of the HIV *pol* gene, thus HIVDR mutations that are commonly detected are in the RT and PR regions. Evolution of drug-resistant HIV subpopulations can significantly compromise the ability of ARVs to suppress viral replication. Once resistant viral strains emerge and replicate, they can persist indefinitely either as circulating virus or integrated into memory T-lymphocyte genomes as proviral DNA. Not only can these drug-resistant viral strains cause HIVDR in patients who acquired them, but they also can be transmitted to newly HIV-infected individuals, leading to compromised ART efficacy in these patients. Two established methods are available for HIVDR testing: genotypic and phenotypic testing. Both tests are complex and expensive. In resource-rich countries, monitoring patients on ART for acquired HIVDR and detection of transmitted HIVDR in recently HIV-infected individuals has become the standard of care (*17*). HIVDR testing has been demonstrated to improve survival and the immune response when used to guide clinical decision-making for patients who have failed one or more previous regimens. Owing to limited and/or lack of infrastructure, trained personnel, and high cost, individual HIVDR testing is not performed routinely in most resource-limited settings. WHO recommends population-based HIVDR monitoring and surveillance to prevent the development and transmission of HIVDR in countries rapidly scaling-up ART programmes and to ensure that ART regimens selected for inclusion in national guidelines, and the ART programmes administering them, continue to be effective (*18–20*).

15.3.4.1 Genotypic HIVDR testing

Genotypic HIVDR testing evaluates the nucleotide sequences from which the amino acids of HIV PR and RT enzymes are deduced. The amino acid sequences of the RT and PR regions of the HIV *pol* gene are compared to those of a wild-type reference viral stain or a subtype-specific consensus reference sequence and any amino acid changes recorded as a change in the amino acid at a specific codon. Mutations are described in a standard format based on the numeric position of the mutant codon in the amino acid sequence of PR or RT. For instance, a change from methionine (M) to valine (V) at position 184 of RT is described as M184V. The letter to the left of the number represents the amino acid in that position in the reference RT and the letter to the right displays the comparable amino acid associated with the mutation in the tested HIV strain. Genotypic testing has been shown to be highly reproducible and sensitive, and provides a complete and accurate genetic sequence of the domains of interest. Studies have indicated that genotypic testing can identify HIVDR mutations presented at about 20% in circulating quasispecies.

The commercially available United States of America Food and Drug Administration (FDA)-approved genotypic tests, TRUGENE® and Viroseq® genotyping systems, were designed and approved for genotyping HIV-1 subtype-B viruses, which are the dominant strains circulating in Europe and North America. Although these tests have been used for HIV-1 non-B subtype genotypic testing, their performance characteristics have not been evaluated fully and test results vary. Laboratories around the world have developed and validated genotypic tests that were designed for genotyping diverse HIV-1 group M subtypes and circulating recombinant forms (CRFs) (*21*, *22*).

15.3.4.2 Phenotypic HIVDR testing

In vitro phenotypic testing uses the HIV-1 RT and PR regions of the *pol* gene derived from an HIV-infected individual and incorporates these gene regions to generate a recombinant virus. The susceptibility of the recombinant virus then is determined by susceptibility testing in the presence of various concentrations of a relevant drug. The results are expressed as susceptibility fold changes in the 50% inhibitory concentrations (IC_{50}) compared to cut-off values generated by reference

wild-type virus. There are several limitations to phenotypic testing. Notably, phenotypic testing may not predict clinical outcome adequately if there is a mixed population of wild-type and mutant viral strains present in the viral quasispecies. In addition, phenotypic testing is available only in a limited number of laboratories that use different methods and may result in discordant results. More importantly, the cost of phenotypic testing currently is at least three times higher than genotypic testing. Because of these limitations, phenotypic testing is not recommended for routine population-based HIVDR surveillance purposes.

15.3.4.3 Specimen types for HIVDR testing

The specimen type routinely used for genotypic testing and considered to be the "gold standard" is plasma. The two FDA-approved genotypic tests require plasma for testing. WHO also recommends that plasma be used for acquired HIVDR monitoring surveys in patients who have been treated with first-line ARVs for 12–15 months (*23*). In addition, many studies have evaluated DBS for HIVDR testing in resource-limited settings and DBS has been used successfully in genotypic testing in newly HIV-diagnosed and treatment-naive populations for transmitted HIVDR in recent years. WHO also recommends that DBS be used for surveys of transmitted HIVDR in ARV-naive populations and for surveys of acquired HIVDR in patients initiating ART (*23–25*). While there are important advantages to using DBS for HIVDR testing, including easier sample collection, transportation, and storage without the need for a cold chain (*21*, *22*, *26–28*), limitations exist for the use of DBS for samples collected from patients treated with first-line ARVs participating in surveys of acquired HIVDR at 12 months after the initiation of ART (*23*, *29*). This is due mainly to lower sample input for extraction, lower viral load levels in most treatment-experienced patients, and lower genotyping testing sensitivity in most in-house genotyping assays. However, recent results from studies conducted in resource-limited settings using second-generation real-time PCR-based viral loading tests have revealed that viral load measurement with DBS for patients treated with first-line ARVs may be a viable alternative for viral load measurement (*30–34*). By combining second-generation real-time PCR-based viral load tests with a few sensitive in-house genotyping

assays (*21*, *22*), monitoring of treatment-experienced patients for HIVDR using DBS finally may become feasible in resource-limited settings.

15.3.4.4 Quality-assured genotyping results

As with any molecular technique, HIVDR genotypic testing is prone to cross-contamination if the test is not performed in the manner and laboratory facilities for which they are designed. To standardize HIVDR genotypic testing and ensure the quality of genotyping data in resource-limited settings, WHO/ResNet has developed an HIV drug resistance laboratory strategy (*35*). Genotyping laboratory strategies, including external quality assessment programmes have been developed and implemented in all WHO-accredited drug resistance laboratories, and only WHO-accredited genotyping laboratories have been performing tests and generating data for HIVDR monitoring and surveillance purposes. The WHO HIV/AIDS Department HIV drug resistance team, in collaboration with Drug Resistance Laboratory at the International Laboratory Branch, Division of Global HIV/AIDS, CGH, CDC, has also developed an HIVDR laboratory training package. All these quality measures have ensured the quality of genotyping data.

15.4 References

1. *UNAIDS world AIDS day report 2011.* Geneva, Joint United Nations Programme on HIV/AIDS, 2011.

2. WHO, UNAIDS, CDC. *HIV rapid testing: training package.* Atlanta, GA, USA, Centers for Disease Control and Prevention, 2006.

3. WHO, UNAIDS. *HIV assays: operational characteristics (phase 1). Report 14, simple/rapid tests.* Geneva, World Health Organization, 2004.

4. USAID. HIV/AIDS rapid test kits: process for USAID approval and technical guidance. Washington, DC, United States Agency for International Development, 2010 (http://transition.usaid.gov/our_work/global_health/aids/TechAreas/treatment/testkit_explain.pdf, accessed 5 April 2013).

5. Bhowan K et al. Identifying HIV infection in South African women: how does a fourth generation HIV rapid test perform? *African Journal of Laboratory Medicine*, 2011, 1(1). http:// dx.doi.org/10.4102/ajlm.v1i1.4.

6. Leelawiwat W et al. Dried blood spots for the diagnosis and quantitation of HIV-1: stability studies and evaluation of sensitivity and specificity for the diagnosis of infant HIV-1 infection in Thailand. *Journal of Virological Methods*, 2009, 155(2):109–117.

7. Ou CY et al. Early diagnosis of HIV infection in the breastfed infant. *Advances in Experimental Medicine and Biology*, 2012, 743:51–65.

8. Ou CY et al. Identification of HIV-1 infected infants and young children using real-time RT PCR and dried blood spots from Uganda and Cameroon. *Journal of Virological Methods*, 2007, 144(1–2):109–114.

9. Facente SN et al. Performance of risk-based criteria for targeting acute HIV screening in San Francisco. *PLoS One*, 2011, 6(7):e21813.

10. Pilcher CD et al. Approaching "HIV elimination": interventions for acute HIV infection. *Current HIV/AIDS Report*, 2006, 3(4):160–168.

11. UNAIDS/WHO Working Group on Global HIV/AIDS/STI Surveillance. *Guidelines for using HIV testing technologies in surveillance: selection, evaluation and implementation, 2009 update.* Geneva, World Health Organization, 2009.

12. Granade TC et al. Factors influencing HIV-1 banding patterns in miniaturized western blot testing of dried blood spot specimens. *Journal of Immunological Methods*, 1992, 154(2):225–233.

13. *Guide to implementation of services for early diagnosis of HIV in infants in resource-limited settings.* Atlanta, GA, USA, Centers for Disease Control and Prevention, 2009 (http://www. womenchildrenhiv.org/wchiv?page=ch-09-00-eid, accessed 5 April 2013).

14. *Antiretroviral therapy for HIV infection in adults and adolescents: recommendations for a public health approach—2010 revision.* Geneva, World Health Organization, 2010.

15. Westerman LE et al. A quality management systems approach for CD4 testing in resource-poor settings. *American Journal of Clinical Pathology*, 2010, 134(4):556–567.

16. *Laboratory guidelines for enumerating CD4 T lymphocytes in the context of HIV/AIDS.* New Delhi, World Health Organization Regional Office for South-East Asia, 2007 (http://www.who.int/hiv/amds/LaboratoryGuideEnumeratingCD4TLymphocytes.pdf, accessed 5 April 2013).

17. Hammer SM et al. Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. *JAMA*, 2008, 300(5):555–570.

18. Bennett DE et al. The World Health Organization's global strategy for prevention and assessment of HIV drug resistance. *Antiviral Therapy*, 2008, 13(Suppl 2):1–13.

19. Bennett DE et al. Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. *Antiviral Therapy*, 2008, 13(Suppl 2):25–36.

20. Jordan MR et al. World Health Organization surveys to monitor HIV drug resistance prevention and associated factors in sentinel antiretroviral treatment sites. *Antiviral Therapy*, 2008, 13(Suppl 2):15–23.

21. Yang C et al. Development and application of a broadly sensitive dried-blood-spot-based genotyping assay for global surveillance of HIV-1 drug resistance. *Journal of Clinical Microbiology*, 2010, 48(9):3158–3164.

22. Buckton AJ et al. Development and optimization of an internally controlled dried blood spot assay for surveillance of human immunodeficiency virus type-1 drug resistance. *Journal of Antimicrobial Chemotherapy*, 2008, 62(6):1191–1198.

23. *WHO manual for HIV drug resistance testing using dried blood spot specimens.* Geneva, World Health Organization, 2010 (http://www.who.int/hiv/topics/drugresistance/dbs_protocol.pdf, accessed 5 April 2013).

24. Somi GR et al. Surveillance of transmitted HIV drug resistance among women attending antenatal clinics in Dar es Salaam, Tanzania. *Antiviral Therapy*, 2008, 13(Suppl 2):77–82.

25. Kamoto K, Aberle-Grasse J, Malawi HIV Drug Resistance Task Force. Surveillance of transmitted HIV drug resistance with the World Health Organization threshold survey method in Lilongwe, Malawi. *Antiviral Therapy*, 2008, 13(Suppl 2):83–87.

26. Ziemniak C et al. A sensitive genotyping assay for detection of drug resistance mutations in reverse transcriptase of HIV-1 subtypes B and C in samples stored as dried blood spots or frozen RNA extracts. *Journal of Virological Methods*, 2006, 136(1–2):238–247.

27. Bertagnolio S et al. HIV-1 drug resistance surveillance using dried whole blood spots. *Antiviral Therapy*, 2007, 12(1):107–113.

28. McNulty A et al. Evaluation of dried blood spots for human immunodeficiency virus type 1 drug resistance testing. *Journal of Clinical Microbiology*, 2007, 45(2):517–521.

29. Bertagnolio S et al. Dried blood spots for HIV-1 drug resistance and viral load testing: a review of current knowledge and WHO efforts for global HIV drug resistance surveillance. *AIDS Reviews*, 2010, 12(4):195–208.

30. Johannessen A et al. Dried blood spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania. *Clinical Infectious Diseases*, 2009, 49(6):976–981.

31. Mbida AD et al. Measure of viral load by using the Abbott Real-Time HIV-1 assay on dried blood and plasma spot specimens collected in 2 rural dispensaries in Cameroon. *Journal of Acquired Immune Deficiency Syndromes*, 2009, 52(1):9–16.

32. Brambilla D et al. Multicenter evaluation of use of dried blood and plasma spot specimens in quantitative assays for human immunodeficiency virus RNA: measurement, precision, and RNA stability. *Journal of Clinical Microbiology*, 2003, 41(5):1888–1893.

33. Garrido C et al. Correlation between human immunodeficiency virus type 1 (HIV-1) RNA measurements obtained with dried blood spots and those obtained with plasma by use of Nuclisens EasyQ HIV-1 and Abbott RealTime HIV load tests. *Journal of Clinical Microbiology*, 2009, 47(4):1031–1036.

34. Lofgren SM et al. Evaluation of a dried blood spot HIV-1 RNA program for early infant diagnosis and viral load monitoring at rural and remote healthcare facilities. *AIDS*, 2009, 23(18):2459–2466.

35. *WHO/HIVResNet HIV drug resistance laboratory strategy.* Geneva, World Health Organization, 2010 (http://www.who.int/hiv/pub/drugresistance/hiv_reslab_strategy.pdf, accessed 5 April 2013).

Annex 1

Microscopy and principles of staining

A1.1 Introduction

Microscopy for sexually transmitted infections (STIs) provides a simple, rapid, and inexpensive test that can be used near to the patient (*1*). It can be sensitive and specific, is ideal for screening, and often can give a presumptive diagnosis that guides treatment and, hence, breaks the chain of transmission. Interpretation of the microscopic image is a skill that requires training, including good working knowledge of the microscope (*1*).

A1.2 Preparation of smears for microscopy

A good-quality specimen from the appropriate site taken with a suitable swab or loop is an essential requirement for good microscopy technique. It is important the slide is clean and the specimen is placed on the correct side if it is frosted on one side. For wet preparations, sufficient sample should be placed onto the slide with a drop of saline if necessary and a coverslip placed carefully on the top. There should be sufficient liquid to spread under the coverslip only and should be examined immediately under low magnification without oil immersion. For smears for Gram staining, the specimen should be rolled evenly across the slide and fixed either by heat or alcohol, stained well, and examine under oil immersion.

A1.3 Light transmission microscopy

The key to good microscopy is to have the microscope set up correctly and to sit comfortably at the bench with the back supported and the eyepieces level at eye height. The microscopist should have a good knowledge of the individual components of the microscope (Fig. A1.1). The microscope should be kept clean and covered when not in use and serviced regularly. It is good practice to rack the stage away from the lens and to use the lowest power that gives the best image.

Procedure for setting up the light microscope:
1. Switch on the microscope
2. Rack the stage down, place slide
3. Swing in the 10× objective
4. Looking at the stage, rack stage up (thinking about the working distance)
5. Adjust intraocular distance and focus on specimen
6. Close the field diaphragm and open condenser fully
7. Move the condenser up or down until the edge is sharp
8. Using centring screws, centre the image
9. Open field diaphragm (take care of light intensity)
10. Readjust light intensity for comfortable viewing
11. Close condenser diaphragm slowly until the image sharpens and the glare disappears
12. Readjust light intensity if required.

Key definitions

Magnification—the number of times the length, breadth, or diameter but not the area of an object is multiplied.
a. "Useful magnification" is where the image is sharp and fine detail revealed, usually at a maximum of 1000×.
b. "Empty magnification" is seen at a certain power where image sharpness is lost and no further detail revealed.

Magnification = power of eyepiece × power of objective × power of inclined head. It is often better to use a lower magnification to see object detail clearly and give a larger field of view.

Resolution—the ability to reveal closely adjacent structural details as separate and distinct.

Definition—the capacity of a lens to render a clear, distinct outline of the object image.

Field diaphragm—protects the object from excess heat and light that is not necessary for image formation.

Condenser or aperture diaphragm—contributes to the resolution and contrast of the image. Do not use for light brightness control.
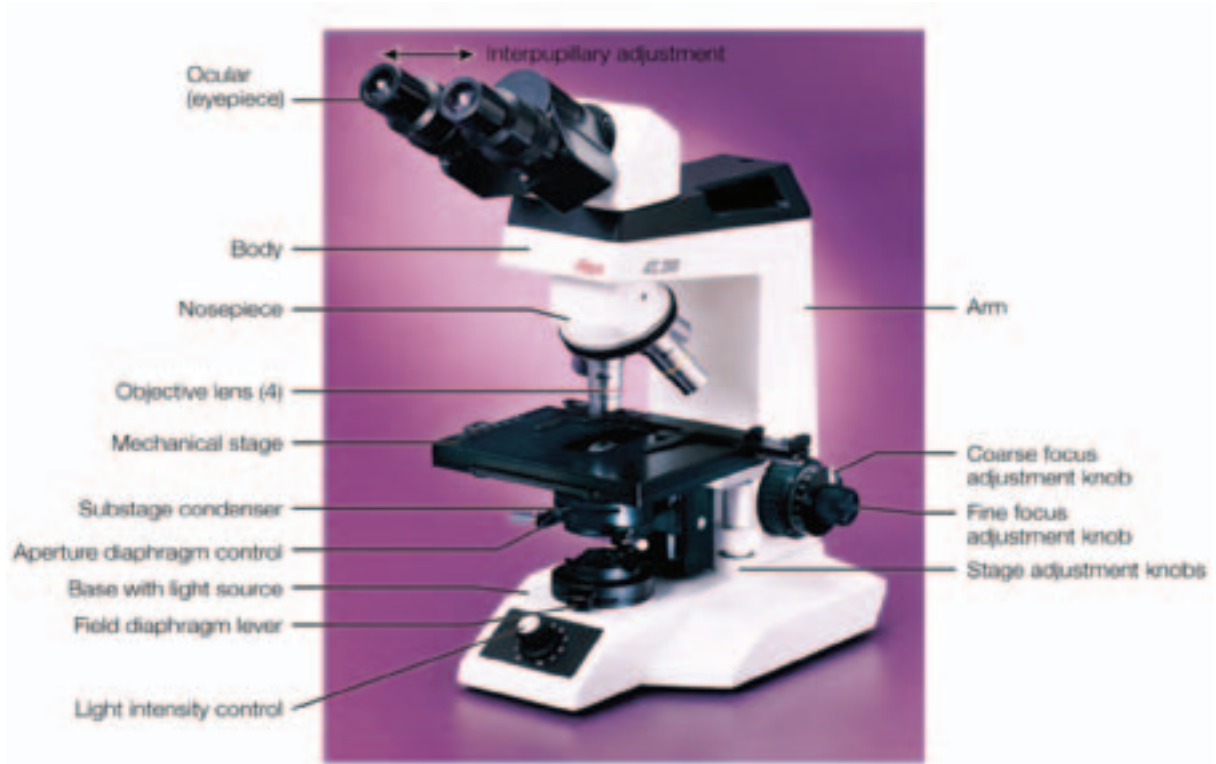
Figure A1.1
Components of a light transmission microscope
Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

Condenser—focuses light on the object. Modern instruments have a "flip-out" or auxiliary lens for use with objectives of 20× and greater to avoid continual resetting of the optics.

Problems that may occur with light microscopy:
- object fuzzy and unable to get clear focus
  - oil on the lens, which should be cleaned with a recommended solvent
  - oil may be behind the lens—may need to contact the manufacturer
  - the condenser lens may have oil on it
  - slide may have been wet before oil was applied
  - different batches of oil may have been mixed together
  - coverslip may be too thick
  - slide is not spread sufficiently and is too thick
  - the correct side of the slide may not be in use
  - coverslip may be dirty with oil or finger marks
  - dried oil on the slide
- bubbles moving across the slide
  - different batches of oil may have been mixed

- water still on slide when oil was applied
- air bubbles may have formed when oil was applied
- uneven illumination
  - microscope not set up correctly; condenser not centred
  - lens turret may be aligned incorrectly
- extraneous particles appear
  - dust may be present on eyepiece, lens, slide, or coverslip, which should be removed carefully.

A1.4 Fluorescence microscopy

Fluorescence microscopy can be used to enable the visualization of some bacteria and viruses not easily visible by light microscopy following staining by a specific antibody attached to a fluorochrome, immunofluorescence. It can be useful for the detection for microbial agents such as *Treponema pallidum, Chlamydia trachomatis*, and herpes simplex virus, or to aid identification using a species-specific antibody, such as for *Neisseria gonorrhoeae*. Fluorescence microscopy requires training to provide expertise for interpretation,

particularly in distinguishing artificial background fluorescence from specific fluorescence. The microscope is best kept and used in a room that can be darkened.

In fluorescence microscopy, the sample is stained using a fluorochrome and then is illuminated with light of a wavelength that excites the fluorochrome in the sample; this is captured by the objective lens. For general purposes for STIs, this can be achieved with an attachment to a transmitted light microscope, which houses a lamp (mercury-vapour or tungsten-halogen) and has two filters: an illumination (or excitation) filter, which ensures the illumination is near monochromatic and at the correct wavelength; and a second emission (or barrier) filter, which ensures none of the excitation light source reaches the detector.

The following problems can occur with fluorescence microscopy:

- image is not visible or is darker than expected if microscope is set up incorrectly or the shutter knob is closed;
- specimen is emitting secondary or autofluorescence caused by non-specific staining of the specimen or dirty objective lens;
- the image is illuminated unevenly due to the components of the microscope not being properly aligned;
- there is excessive glare in the eyepiece because the correct filters are not present;
- rapid bleaching of the specimen when being viewed.

A1.5 Dark-field microscopy

Dark-field microscopy differs from transmitted light microscopy in that only light rays striking organisms or particles at an oblique angle enter the microscope objective, giving rise to bright, white luminescent bodies against a black background (Fig. A1.2). Dark-field microscopy for detection of *T. pallidum* for the diagnosis of syphilis has to be performed by well-trained and experienced personnel who are able to adjust the microscope correctly and to differentiate *T. pallidum* from non-pathogenic treponemes and other spiral organisms commonly found on genital and anal mucous membranes. As the oral cavity often is colonized by spirochaetes other than treponemes, dark-field examination of material from oral lesions is not recommended.

transmitted   dark-ground
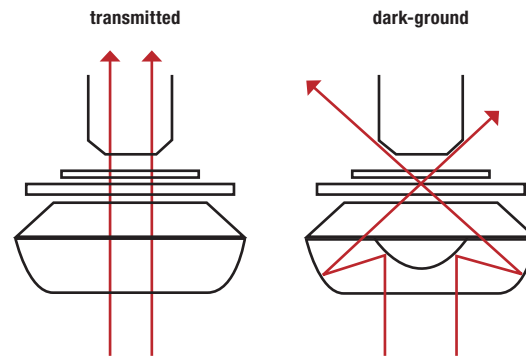
Figure A1.2
Comparison of light path between a transmitted light microscope and a dark-field (dark-ground) microscope
Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

Procedure for use of the dark-field microscope:

1. Make a thin preparation (Chapter 10) using a thoroughly cleaned slide and coverslip. Too much material will negate the effect.
2. Rack the condenser down and place a drop of oil on top of the lens. Take care to avoid trapping air bubbles.
3. Place the slide on the stage and slowly rack up the condenser until the oil just touches the slide. A brief flash of light will be seen. Take care not to allow bubbles to form.
4. Focus on the object with a low power objective (10×). If the condenser is correctly focused, a small point of light will illuminate the object against a dark background. If a hollow ring of light is seen, the condenser needs to be adjusted.
5. With the centring screws, adjust the condenser until the point of light is in the middle of the field.
6. Place a drop of oil on the coverslip and focus the object with the oil-immersion lens. If an objective with an iris diaphragm is used, slight adjustment may be necessary. If the objectives are not par-central, then centring of the condenser may be required.
7. Dark-field microscopy is best conducted in a darkened room.

The following problems may occur with dark-field microscopy:

- Slides or coverslips may be too thick
- Preparation has too many air bubbles
- Condenser may not be correctly focused or centred

- Lighting may not be sufficiently intense
- Field of view is too small (open field diaphragm)
- Centre of the field of view is dark (the slide is too thick or the condenser is in the wrong position).

A1.6 Phase contrast microscopy

Phase contrast microscopy is seldom available or used but has some advantages when examining living, unstained specimens because they have the same refractive index as mounting fluid and internal detail may be difficult to see. Unstained objects may be considered as having the properties of a diffraction grating. Light passing through different parts of the object is affected and its phase slightly changed, but this is not discernible to the eye. However, by optical means these phase differences may be made visible as light and dark areas.

The following are needed for phase contrast microscopy:

1. a special condenser carrying a series of annular diaphragms, the annulus size varying with the numerical aperture of the objective used;
2. special phase objectives containing a phase plate. This plate consists of a disk of glass having a circular trough etched in it and of such depth that light that is passing through this portion has a phase difference of one quarter of a wavelength compared with the rest of the plate.

Procedure for use of the phase contrast microscope:

1. Prepare a thin, not too crowded, specimen slide and swing in the objective (objectives are labelled Ph, Phaco, or similar).
2. Rotate the appropriate annulus for that objective into position in the condenser.
3. Check that the annulus and phase plate are in the correct position using the telescope supplied with the system.
4. Remove an eye-piece and insert the telescope. Images of the annulus and phase plate should be coincident. If not, adjust with the condenser centring screws.
5. Replace the eye-piece and examine the specimen.
6. All powers of objective may be used provided that they are fitted with a phase plate and that there is an appropriate annulus in the condenser.

The following problems can occur with phase contrast microscopy:

- condenser and objective not matched by Ph code;
- phase plate is not aligned so it is necessary to check using the telescope;
- the field diaphragm is not focused on the specimen surface, which should be solved by moving the condenser up or down.

A1.7 Gram stain

This stain was first described in 1884 by Gram and demonstrates the morphology of bacteria and fungi. It divides bacteria into two main groups: those that appear positive or those that appear negative. Each of these then can be further divided into cocci (spheres) or bacilli (rods), resulting in four groups. Many bacteria that appear very distinctive, such as *N. gonorrhoeae,* are Gram-negative diplococci, but it is important to remember that the Gram stain reaction only gives a presumptive identification. Other identification methods are necessary to classify any bacteria. Fungi appear as Gram-positive.

A1.7.1 Principle of the Gram stain

The Gram stain reflects differences in the bacterial cell wall structure in that Gram-positive bacteria have large amounts of peptidoglycan in the cell wall, which retains the purple stain (Fig. A1.3a), whereas Gram-negative bacteria have a complex cell wall with less peptidoglycan, which does not retain the purple stain but takes up the pink counterstain (Fig. A1.3b).

A1.7.2 Method of use

A fixed smear is flooded with crystal violet for 30 seconds, during which all bacteria take up this stain (Fig. A1.4). The smear then is gently washed in tap water and flooded with Lugol's iodine for a further 30 seconds, which acts as a mordant and fixes the purple stain in the cell membrane.

After washing with water, a decolorizer is used to remove excess stain; it is at this stage that the crystal violet/iodine complex is washed out of the cell membrane of Gram-negative bacteria as there is insufficient peptidoglycan to retain it. A number of decolorizers are used, ranging from alcohol, which is slow and gentle, to

Figure A1.3A
Structure of a Gram-positive cell wall
Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

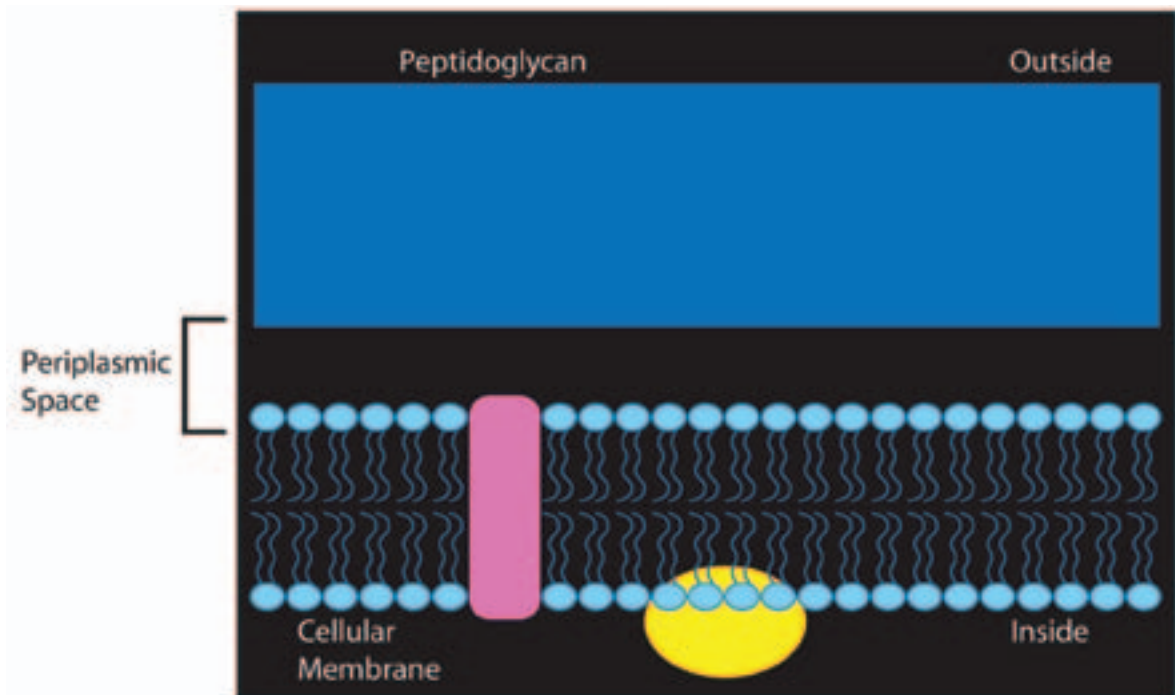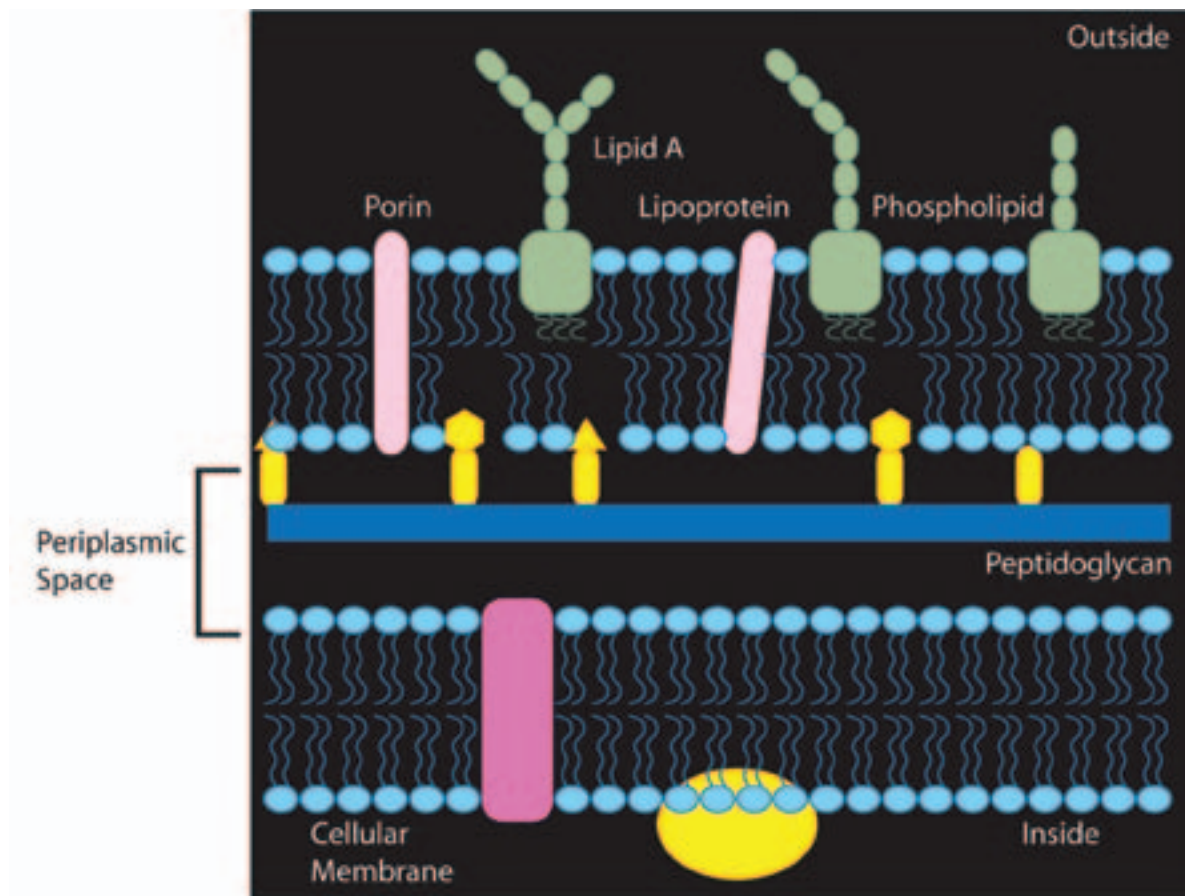**Figure A1.3B**
Structure of a Gram-negative cell wall
Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

acetone, which is harsh and fast. The time the decolorizer is left on the smear varies from a few minutes for alcohol to just a few seconds for acetone. It is advisable to hold the smear over the sink and to allow the decolorizer to run over the smear. The purple complex will run out of the smear and as soon as this stops, the smear should be washed with water. It is easy at this stage to over-decolorize the smear, particularly if acetone is used; many find a mixture of acetone and alcohol (1:1) a good combination, as the speed of the acetone is slightly tempered by the alcohol.

After decolorization, the smear is flooded with a counterstain for approximately 1 minute and it is at this stage that Gram-negative bacteria take up the pink stain. There are again a number of counterstains used: safranin, neutral red, and carbol fuchsin. Safranin and neutral red are good stains for smears that contain polymorphs, as these give good definition of the cell structure, whereas carbol fuchsin is preferable for bacteria.

The individual times used for each of the stains can vary, but the absolute times used are not crucial. However, it is important to remember that while the primary stain (crystal violet) acts almost instantly, counterstaining is a slower process and requires more time. It is advisable to leave the counterstain on for twice the time for the primary stain.

A1.7.3 Problems and remedies

Stains that are well-prepared and stored correctly are essential for producing a well-stained smear. The individual stains are available commercially and it is now relatively unusual for these to be prepared from the powder. However, even when stains are purchased either ready for use or as concentrated stain to be diluted for use, it is important to store the stains correctly. Stains should be stored in clean bottles and, if the bottles are reusable, any residual stain should be discarded and then rinsed with water before refilling. Inappropriately stored stains can result in stain deposit either over the entire smear or adhering to epithelial cells, which could be confused with "clue cells".

The smear needs to be of an appropriate thickness; a smear that is too thick will retain the stain and prevent differentiation between Gram-negative and -positive

Gram positive   **Gram negative**

Fixation

Crystal violet

Iodine treatment

Decolorisation

Counter stain safranin
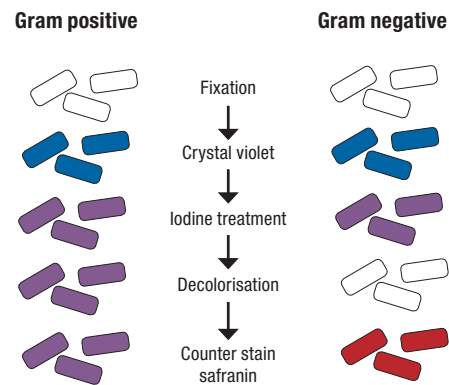
Figure A1.4
Reaction of Gram-positive and Gram-negative bacteria to the different stages of the Gram stain
Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

organisms, and a smear that is too thin and has insufficient material may not give a true representation of the sample.

The decolorizing step is the most crucial step in the Gram stain and over- or under-decolorization will result in bacteria being categorized incorrectly as either Gram-negative or Gram-positive, respectively.

A1.8 Methylene blue stain

The use of methylene blue as a single stain is very useful in settings where there are either inadequate facilities or resources for using the Gram stain. It will show the morphology of both bacteria and host cells but lacks the differentiation obtained with the Gram stain. Its primary use is for staining smears for the presumptive diagnosis of gonorrhoea and gives a distinctive picture that has a high predictive value in high-risk patients.

A1.9 Giemsa stain

Giemsa stain is a differential stain used for the diagnosis of donovanosis. It is a mixture of methylene blue, eosin, and azure B and is available commercially. A smear is prepared, fixed in methanol for 30 seconds, immersed in freshly prepared 5% Giemsa stain for 20–30 minutes, washed, and allowed to dry.

A1.10 Immunofluorescence

Immunofluorescence is a technique that uses the highly specific binding of an antibody to its antigen to label

specific proteins or other molecules within the cell. A sample is treated with a primary antibody specific for the molecule of interest. A fluorophore can be conjugated directly to the primary antibody. Alternatively, a secondary antibody conjugated to a fluorophore, which binds specifically to the first antibody, can be used.

A1.11 Health and safety

There should be a nominated person responsible for health and safety in each workplace, either clinic or laboratory. The staining methods should be described in standard operating procedures, which should be regularly updated and have been read and understood by all staff.

A number of ground rules should be in place:
- No eating, smoking, or drinking in a laboratory area.

- Provide good ventilation in the area used for staining to avoid inhalation of fumes and fire hazards.
- Wear gloves and appropriate laboratory coats when handling the individual reagents used for staining, as these are known to be toxic; some Gram stains are carcinogenic.
- Store stain carefully to prevent spillage.
- Take care in disposing slides and coverslips from infected patients.
- Store flammable material in a specific cupboard and keep away from heat sources.

A1.12 Reference

1. British Association for Sexual Health and HIV (BASHH). Microscopy for Sexually Transmitted Infections (http://www.bsig-resources.org.uk/).

Annex 2

Rapid point-of-care test principles

A2.1 Introduction

The 2004 World Health report cites unaffordability and inaccessibility as two major reasons why health services fail (*1*). In countries with a high burden of sexually transmitted infections (STIs), laboratory services for STIs are either not available or, where limited services are available, patients may not be able to physically access or to pay for these services. The WHO recommends the use of syndromic management where patients are treated for all the major causes of a particular syndrome (*2*). Syndromic management of STIs works well for urethral discharge, pelvic pain, and genital ulcer disease, but evaluations of the WHO flowcharts have shown that the algorithm for vaginal discharge lacks both sensitivity and specificity for the identification of women with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection. Simple, affordable, rapid tests that can be performed at the point-of-care (POC) and enable treatment and case management decision to be made are urgently needed for these infections. Simple rapid POC tests are needed not only to increase the specificity of syndromic management, and reduce over treatment of genital gonococcal and chlamydial infections but also to screen for asymptomatic STIs (*3*, *4*, *5*). A mathematical model estimated that a test for syphilis that requires no laboratory infrastructure could save more than 201 000 lives and avert 215 000 stillbirths per year worldwide. A similar test could save approximately 4 million disability-adjusted life years (DALYs), avert more than 16.5 million incident gonorrhoea and chlamydial infections and prevent more than 212,000 HIV infections per year (*6*).

A2.2 Characteristics of the ideal rapid point-of-care (POC) test

If diagnosis can be made at the POC, treatment, counseling and partner notification can be started without delay. The acronym, ASSURED (see Box 1), was coined, at a 2003 meeting of the WHO Special Programme for Research and Training in Tropical Diseases (WHO/TDR), to define the ideal characteristics

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Box 1: The ideal rapid test—ASSURED criteria

A = <u>A</u>ffordable

S = <u>S</u>ensitive

S = <u>S</u>pecific

U = <u>U</u>ser-friendly (simple to perform in a few steps with minimal training)

R = <u>R</u>obust and rapid (can be stored at room temperature and results available in <30 minutes)

E = <u>E</u>quipment-free or minimal equipment that can be solar- or battery-powered

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of a rapid test that can be used at the POC within all levels of a health care system (*7*).

Rapid versus point-of-care (POC) tests

Rapid tests generally have been defined as tests that give a result in <30 minutes. POC tests can be defined as tests that are simple and can be performed at all health care settings, especially at primary health care settings, with minimal training and no equipment (or small equipment that is solar- or battery-powered). Historically, Gram stained microscopy, wet prep, pH, the amine or Whiff test, and the rapid plasma reagin (RPR) or Venereal Diseases Research Laboratory (VDRL) tests are considered rapid tests and, in principle, can provide immediate results to guide treatment. However, some of these tests depend on a source of electricity to operate equipment such as a centrifuge or microscope, which means they cannot be used at POC settings without a source of electricity. The RPR test for syphilis is a case in point. It only requires 8–10 minutes to perform, but testing is often batched and, therefore, cannot be used to facilitate immediate testing and treatment. In addition, the RPR reagent requires refrigeration, a centrifuge is

needed to separate serum from whole blood, and a rotator is required for mixing the reaction. Hence the RPR, in spite of its name, is neither used as a rapid test nor could it be considered a POC test as it cannot be performed in settings where there is no electricity.

Simple rapid POC treponemal tests, developed as immunochromatographic tests (ICTs) in a lateral flow or dipstick format, have been shown to have acceptable performance (8). These tests can be used with whole blood obtained by finger pricks, stored at room temperature for up to 18 months, requires minimal training, and gives a result in 15–20 minutes. Table A2.1 summarizes the differences between two types of rapid tests for the serodiagnosis of syphilis.

A variety of rapid tests is commercially available for the diagnosis of STIs (Table A2.2). Vaginal infections such as candidiasis, bacterial vaginosis or trichomoniasis can be diagnosed with a microscope using Gram-stained smears or wet preparation of vaginal secretions. However, presumptive treatment with metronidazole often is given to patients with vaginal discharge, as microscopy results for bacterial vaginosis, candidiasis, or trichomoniasis are rarely available during a patient's visit to the clinic. Rapid tests that can aid in the diagnosis of bacterial vaginosis are detection of acidic pH using a piece of pH paper or the characteristic amine odour on the addition of potassium hydroxide to vaginal secretions.

Since most rapid test kits do not have internal quality controls and are commonly used outside of laboratory settings, it is critical that control programmes develop quality assurance programmes to assure the quality of test and testing. Special considerations for the use of rapid test kits are listed in Box 2 below.

Box 2: Caution in the use of rapid tests

- **Before use:**
  - note the expiry date of the rapid test kits; do not use beyond the expiry date
  - check that all the items are supplied as stated in the product insert
  - note the appearance of the box to ensure integrity of the box and that the test kits have not been damaged in shipment

- **Use of test kits:**
  - follow instructions in the product insert
  - do not mix reagents from different boxes

- **After use:**
  - ensure proper disposal of sharps and other wastes

Table A2.1: Differences between two types of rapid tests using non-treponemal and rapid treponemal tests as examples

Rapid test e.g. the non-treponemal plasma reagin test (RPR)	Rapid POC test e.g. treponemal test
Use serum or plasma (requires centrifuge)	Use whole blood, serum, or plasma
Reagent require refrigeration	Test kits can be transported and stored at room temperature
Needs equipment (refrigerator, centrifuge, and rotator) and trained personnel	No equipment and minimal training required
Test result available in 8–10 minutes	Test result available in 15–20 minutes
Antibodies associated with a recent infection can be used to distinguish active from previously treated infection and for test of cure	Treponemal antibodies persist for years; measure of exposure cannot be used to distinguish active from previously treated infection

Table A2.2: Rapid tests for the diagnosis of STIs

Tests	STIs	Equipment	Approximate cost (US$)	Comments
Microscopy: • Gram stain • Dark-field (direct or with fluorescent antibody • Wet prep • Clue cells • Nugent score	*Neisseria gonorrhoeae* *Treponema pallidum* *Trichomonas vaginalis* Candidiasis Bacterial vaginosis Bacterial vaginosis	• Microscope • Electricity	$0.50	Sensitivity 95% for urethral infections in symptomatic men but <50% for women
pH	Bacterial vaginosis	none		
Amine (KOH)	Bacterial vaginosis	none	$3–6	
Flocculation: • RPR • VDRL	Syphilis	• Electricity to run centrifuge and rotator • Microscope	$0.20	Result not often given the same day because of batching
Rapid tests (Immunochromato-graphy strips)	*Neisseria gonorrhoeae* *Chlamydia trachomatis* *Trichomonas vaginalis* Syphilis HIV Herpes simplex viruses	none	$6–20 $6–20 $3–5 $0.50–3.00 $5–10 $35	
Enzyme detection	Bacterial vaginosis (*Gardnerella spp.*)	none	$3–6	
NAATs	*Neisseria gonorrhoeae* *Chlamydia trachomatis*	• Real-time PCR assays or isothermal amplification assays	$15–35	These new molecular assays require very little user input and can give results in 15–100 minutes

HIV, human immunodeficiency virus; KOH, potassium hydroxide; NAAT, nucleic acid amplification test; PCR, polymerase chain reaction; RPR, rapid plasma reagin; STI, sexually transmitted infection; VDRL, Veneral Disease Research Laboratory.

A2.3 Principles of rapid point-of-care (POC) test technologies

A2.3.1 Agglutination reactions to detect antigen or antibody

A2.3.1.1 Flocculation

In the RPR test, antibodies to non-treponemal antigens, such as cardiolipin, can be detected in serum samples using an antigen-antibody reaction known as flocculation. A small amount of serum is added inside a circle on the test card. A drop of the test reagent that contains cardiolipin and carbon particles is added to the serum sample. The mixture is stirred or shaken gently for 8 minutes. A positive antigen-antibody reaction results in a lattice formation in which the carbon particles are trapped, as shown in Figure A2.1. The carbon particles help in the visualization of the positive reaction. A homogeneous pattern denotes a negative result. An excess of antibody in a serum sample may result in a false negative result, as the typical antigen-antibody lattice structure cannot be formed. This false negative result is known as a prozone phenomenon.

Instead of carbon particles, some tests use toluidine red dye to improve the visualization of the test results. As the reading of the test result is subjective, it is important that the user is properly trained to perform and interpret the test results.
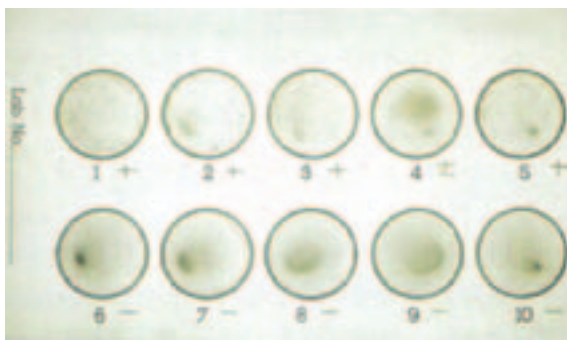
Figure A2.1
Rapid Plasma Reagin (RPR) test
Source: Van Dyck E, Meheus AZ, Piot P. Laboratory diagnosis of sexually transmitted diseases. Geneva, World Health Organization, 1999.

A2.3.1.2 Agglutination reactions using latex particles

Latex particles coated with an antigen, such as treponemal antigens, offer a test that may be easier to interpret compared to the use of carbon particles in the RPR test. A serum sample and coated latex particles are mixed for 8 minutes inside a circle on a card. If non-treponemal antibodies are present in the serum, they will form an agglutination pattern with the coated latex particles, which can be visualized by eye. A smooth pattern denotes a negative result. This test is subject to false negative results because of prozone effect, similar to the RPR.

A2.3.2 Immunochromatographic tests (ICTs)

A2.3.2.1 Lateral flow format

In the design of the ICT for the detection of antibody, dye-labelled antibody (Ab), specific for the test target antigen (Ag), is present on the lower end of the nitrocellulose strip, or in a well provided by a casing covering the strip. Ab, specific for another epitope on the target antigen, is bound to the strip in a thin Test line, and Ab specific for the labelled Ab is bound at the Control line. Figure A2.2A is a schematic that represents four stages in the progression of a specimen (either whole blood, serum or plasma) containing treponemal antibodies through a lateral flow immunochromato-graphic strip. At stage 1 the specimen is dropped into the specimen well, treponemal antibodies, represented by a yellow diamond, flow from the specimen well into the sample pad. At stage 2 the antibodies flow through the conjugate pad and combine with colloidal gold-labelled antigen and form a gold antigen-antibody complex. At stage 3, as the complex cross the test line, it combines with the antigen immobilised on the Test line and when sufficient labeled antigen accumulates, the dye labels will become visible to the naked eye as a narrow red line (the Test line). At stage 4 free conjugate combines with the antibody immobilised on the Control line turning it red.

If a specimen does not contain any treponemal antibodies, it would flow through the conjugate pad and the Test line without turning the line red. The free conjugate that flow through with the specimen would just turn the Control line red. If a specimen did not migrate from the specimen well, no lines would appear in the Test line or Control line indicating an invalid assay (Fig. A2.2B).
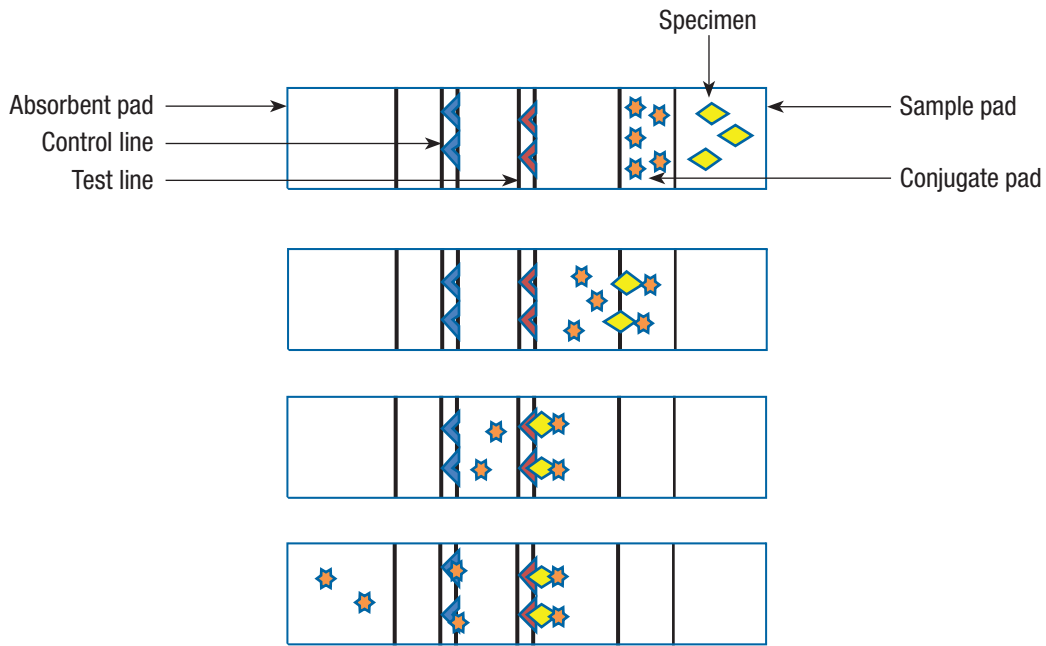
Figure A2.2A
Schematic for a positive syphilis test in a lateral flow immunochromatographic format

Reading test results:

negative

A negative test result showing only the control line (C) appearing in the window.

positive

A positive test result showing both the test (T) and control lines appearing in the window.

indeterminate

An invalid result showing no lines. Either the specimen (S) has failed to migrate along the test strip or the test is no longer working.

Figure A2.2B
Interpretation of a lateral flow assay for the detection of treponemal antibodies from a patient with syphilis

A2.3.2.2 Multiplex ICTs

Lateral flow ICTs that are designed for duplex (HIV and syphilis) or multiplex testing (HIV, syphilis and hepatitis B or C) using a single fingerprick blood sample are now commercially available. Several of these tests are already in clinical trials. A rapid test that can detect both non-treponemal and treponemal antibodies using patented dual path technology has been recently evaluated and found to have acceptable performance (*9*). Several companies are developing oral fluid tests for syphilis alone or in a duplex test with HIV (*10*).

A2.3.2.3 Flow-through format

Instead of a lateral flow strip format, some ICTs are configured as a flow-through device in which the antigens are adsorbed onto a nitrocellulose disc. The serum or plasma then is allowed to flow through the discs and washed before the conjugate for the dye labels are added to display the results.

A2.3.2.4 Rapid test readers

A number of simple inexpensive optical readers or scanners are now commercially available for use with ICTs. The use of these readers removes observer bias, improves sensitivity and allows for quantitation such as for non-treponemal titres.

A2.4 Emerging technologies

While ICTs can detect antibodies with reasonable sensitivity because of the large quantity of antibodies in blood, they tend to be less sensitive for antigen detection. Most rapid tests for the detection of genital chlamydial and gonococcal infections have sensitivities of only 50–70% compared to nucleic acid amplified tests.

A2.4.1 Microfluidic assays

Microfluidic assays that can detect multiple analytes from a single specimen or can reproduce all the performance characteristics of an immunoassay have been developed and applied to HIV and syphilis (*11*, *12*). The assay has few moving parts and the chips cost pennies instead of dollars, making it suitable for resource poor settings. The results can be read with a small battery-powered device. Some of these assays are now in trials in Africa.

In general, these assays work well with antibody detection from blood because of the abundance of target molecules. For other specimens such as urine or swabs, pre-processing of the specimen will be necessary before it can be injected into the test cassette. For antigen detection, getting enough target molecules into the detection zones is a challenge.

A2.4.2 Rapid molecular assays

Recent developments in integrated nucleic acid amplification platforms have given rise to sensitive, specific, user-friendly, sample-in, answer-out diagnostic tools for infectious diseases (*13*). These technologies are promising for multiplex detection of pathogens associated with the major STI syndromes. The GeneXpert assay for the duplex detection of chlamydia and gonorrhoea from a single sample has reasonable performance compared to culture and PCR as reference standards. It can give a result in under two hours. The machine allows random access, which makes it more cost-effective if the equipment can be used for different infectious diseases.
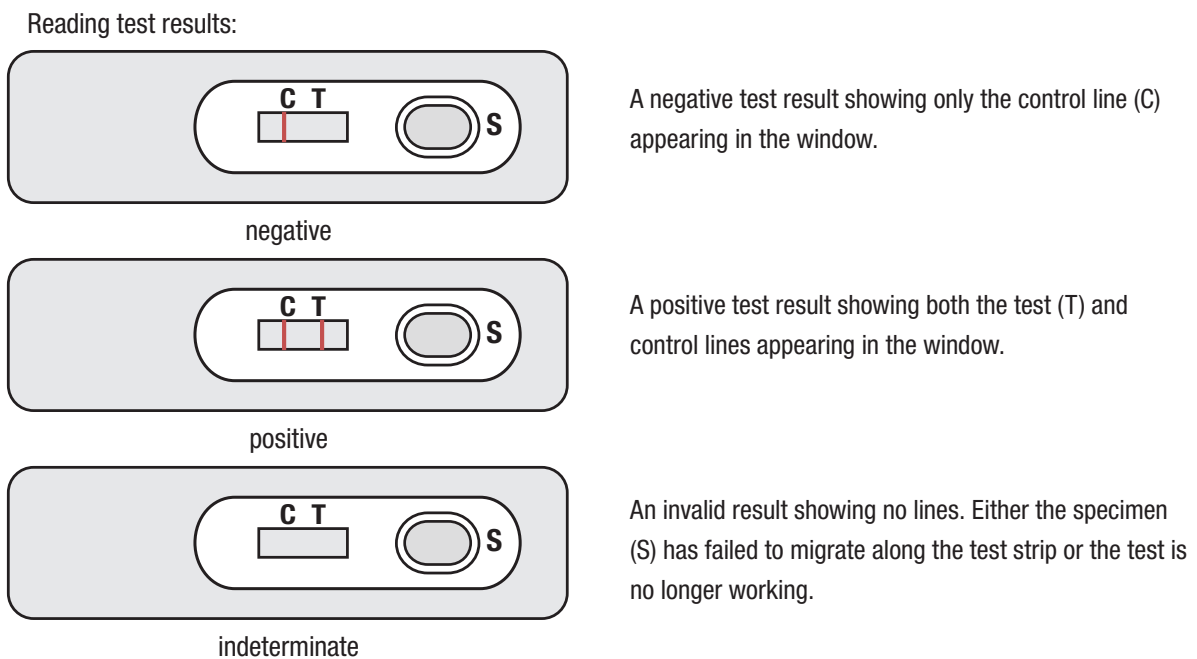
There are a few promising isothermal nucleic acid amplification technologies that are rapid and simple to perform. These are being developed for detection of infectious disease, including chlamydia and gonorrhoea.

Recombinase Polymerase Amplification (RPA) is a 15-minute assay that can achieve the same sensitivity and specificity as most current molecular assays by coupling isothermal recombinase-driven primer targeting of template material with strand-displacement DNA synthesis. This assay uses recombinases that are capable of pairing oligonucleotide primers with homologous sequences in duplex DNA (*14*). Without the need to split double stranded DNA before amplification, this assay can achieve exponential amplification from just a few target copies. All reagents required for this assay are stabilized as dried formulations and can be transported safely without refrigeration. The results can be read on a fluorimeter or visually in a lateral flow strip.

Helicase Dependent Amplification (HDA) is a single-tube isothermal amplification procedure that can detect short DNA sequences (80–120 bp). The results are available in 1.8 hours. However, the reagents need to be stored at −20°C.

Cross Priming Amplification (CPA) is a class of isothermal nucleic acid amplification reactions that utilize multiple primers and probes, one or more of which is a cross primer. The reaction requires a strand displacement DNA polymerase without the need for an initial denaturation step or the addition of a nicking enzyme (*15*). At an assay temperature of 63°C, the formation of a primer-template hybrid at transient, spontaneous denaturation bubbles in the DNA template is favoured over re-annealing of the template strands by the high concentration of primer relative to template DNA. Strand displacement is encouraged by the annealing of cross primers with 5' ends that are not complementary to the template strand and the binding of a displacement primer upstream of the crossing primer. The resulting exponential amplification of target DNA is highly specific and highly sensitive, producing amplicons from as few as four bacterial cells.

Rolling circle amplification (RCA) and **Circle-to-circle amplification (C2CA)** are integrated isothermal amplification platforms and subsequent electrophoretic detection of a specific gene on a poly(methyl methacrylate) microchip (*16*). RCA and C2CA are carried out at 37°C in the sample well of the microchip, and provides a sensitive, fast, high-throughput, and reproducible method for signal amplification.

Rapid advances in sequencing technologies, such as nanopore technology, have made it possible to conduct Genome wide scans rapidly and at relatively low cost. These technologies can be applied to the detection of genes conferring gonococcal resistance.

A2.5 References

1. Devarajan S, Reinikka-Soininen R, World Bank. *Making services work for poor people: world development report 2004.* New York, Oxford University Press for the World Bank, 2003.

2. *Guidelines for the management of sexually transmitted infections.* Geneva, World Health Organization, 2003.

3. Peeling RW et al. Why do we need quality-assured diagnostic tests for sexually transmitted infections? *Nature Reviews. Microbiology*, 2006, 4(12):909–921.

4. Peeling RW et al. Rapid tests for sexually transmitted infections (STIs): the way forward. *Sexually Transmitted Infections*, 2006, 82(Suppl 5):v1–6.

5. Peeling RW. Applying new technologies for diagnosing sexually transmitted infections in resource-poor settings. *Sexually Transmitted Infections*, 2011, 87(Suppl 2):ii28–30.

6. Aledort JE et al. Reducing the burden of sexually transmitted infections in resource-limited settings: the role of improved diagnostics. *Nature*, 2006, 444(Suppl 1):59–72.

7. Kettler H, White K, Hawkes S. *Mapping the landscape of diagnostics for sexually transmitted infections.* WHO/TDR publication. World Health Organization on behalf of the Special Programme for Research and Training in Tropical Diseases, 2004.

8. Mabey D et al. Prospective, multi-centre clinic-based evaluation of four rapid diagnostic tests for syphilis. *Sexually Transmitted Infections*, 2006, 82(Suppl 5):v13–16.

9. Castro AR et al. Novel point-of-care test for simultaneous detection of nontreponemal and treponemal antibodies in patients with syphilis. *Journal of Clinical Microbiology*, 2010, 48(12):4615–4619.

10. Tucker JD, Bien CH, Peeling RW. Point-of-care testing for sexually transmitted infections: recent advances and implication for disease control. *Current Opinion in Infectious Diseases*, 2013, 26(1):73–79.

11. Sorger PK. Microfluidics closes in on point-of-care assays. *Nature Biotechnology*, 2008, 26(12):1345–1346.

12. Chin CD et al. Microfluidics-based diagnostics of infectious diseases in the developing world. *Nature Medicine*, 2011, 17(8):1015–1019.

13. Niemz A, Ferguson TM, Boyle DS. Point-of-care nucleic acid testing for infectious diseases. *Trends in Biotechnology*, 2011, 29(5):240-250.

14. Piepenburg O et al. DNA detection using recombination proteins. *PLoS Biology*, 2006, 4(7):e204.

15. Xu G et al. Cross priming amplification: mechanism and optimization for isothermal DNA amplification. *Scientific Report*, 2012, 2:246.

16. Mahmoudian L et al. Rolling circle amplification and circle-to-circle amplification of a specific gene integrated with electrophoretic analysis on a single chip. *Analytical Chemistry*, 2008, 80(7):2483-2490.
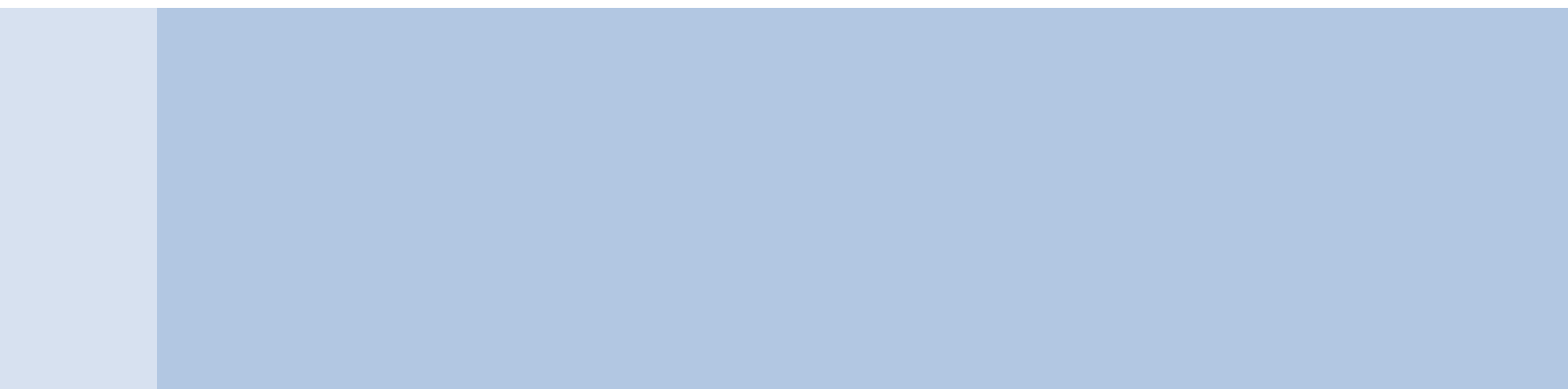
Annex 3

Principles of molecular tests for the diagnosis of sexually transmitted infections

A3.1 Introduction

Sexually transmitted infections (STIs) can be caused by viruses, bacteria, or parasites, and coinfections are not rare. Clinical specimens for STI testing also are sampled from different anatomic sites using various sampling devices and techniques and in different settings where large variations in specimen transport and storage exist, as well as various resources for STI diagnostics. Accordingly, for effective laboratory diagnosis of STIs worldwide, many different methods exist, and these methods might have significantly different sensitivity, specificity, and reproducibility either to test for a single infection, or a wide range of STIs. The historical laboratory methods for the detection of etiological agents of STIs include bacteriological and tissue culture, various serologic testing for the presence of specific antibodies, immunohistochemistry, antigen detection, and microscopy. Similar to the diagnosis of many other infectious diseases, the direct detection of specific nucleic acids from STI pathogens using different nucleic acid amplification tests (NAATs) has become the new "gold standard" for the diagnosis of many STIs due to superior sensitivity and specificity. Many of the NAATs are approved by the United States of America Food and Drug Administration (FDA) or other international, national, or regional regulatory bodies, and commercially available. However, the use of laboratory-developed assays (LDAs) using conventional polymerase chain reaction (PCR)- or real-time PCR-based detection methods for STI diagnosis also are popular, especially, for research and epidemiologic purposes. Currently, many commercially available and even LDA singleplex or multiplex NAATs are in use for STI diagnostics. If any non-FDA-approved NAATs are used, international—such as European Union (EU)—and/or other national regulatory processes should provide safeguards on the quality and performance of the diagnostic NAAT. For diagnosis of STIs, it is recommended to use only internationally approved NAATs. If this is not possible, it is essential that the

proposed NAAT is strictly validated before use according to local requirements. Evaluation should be made comparing against at least one internationally approved NAAT and the assay subsequently should be used with appropriate positive, negative, and inhibition controls. In addition, participation in an appropriate external quality assessment (EQA) system is highly recommended.

In many cases, the NAATs are preferred over conventional diagnostic methods for their superior sensitivity and specificity, shorter turnaround time, high throughput, and opportunity for automation, multiplexing, and quantification. Due to their high sensitivity, the NAATs also are effective in detecting organisms from asymptomatic infections or at early phase of infection (before seroconversion), and can be applied to self-collected and noninvasive specimens, such as vaginal swab and first-catch urine samples.

The molecular diagnostics of STIs using nucleic acid-based tests are very powerful but not without limitations. One of the strengths of NAATs is their ability to detect pathogens without the need of live microorganisms that makes transport and storage of specimens less critical; however, this also constitutes one of the weaknesses of NAATs, e.g. isolates are not available for subsequent antimicrobial susceptibility testing of the target organism or other phenotypic characterization. Accordingly, the NAATs detect nucleic acid from both dead and live target organisms; thus, NAATs are inappropriate for test-of-cure (TOC), unless sufficient time has been allowed for the residual organisms as well as nucleic acids to be cleared from the infected host before TOC sample collection. Furthermore, the close genetic relatedness between the target organism and closely related species, e.g. *Neisseria gonorrhoeae* and commensal *Neisseria spp.*, can cause false-positive results (*1, 2*). False negatives also could emerge because of sequence variation or deletion of the specific target sequence in the organism to be

detected due to mutation, e.g. the Swedish new variant of *Chlamydia trachomatis* (nvCT) (*3*, *4*). The immediate future of the molecular diagnostics of STIs should focus on validating and licensing the use of different assays on rectal and pharyngeal specimens, and on the expansion of current microfluidics and nanotechnology to allow integration of specimen collection, stabilization, and handling, and to develop testing process into affordable, fully automated, high-throughput, and user-friendly systems. In developed countries, point-of-care applications may be a focus of the expansion of the portfolio of STI pathogen detection, incorporation of quantitative detection, and establishment of molecular typing or antimicrobial resistance markers detection in a single multiplexing assay. For resource-limited countries, affordable, sustainable instrumentation with simplistic testing process, less hands-on time, and a closed system to reduce risk of contamination, capability of throughput, and inexpensiveness of test reagents are the most important needs for the field.

A properly designed NAAT theoretically is capable of detecting as little as one target in a sample with very high specificity. However, achieving this level of limit of detection (LOD) requires a series of time-consuming processes for NAAT optimization. In addition, the analytical sensitivity of NAATs also can be influenced by a variety of factors, e.g. inherent factors within the individual clinical specimen such as inhibitors, the specimen type, the copy number of target sequence, the nucleic acid extraction, amplification, and detection technology used. There are five critical stages in nucleic acid-based tests: sample collection, sample stabilization/storage/transport, nucleic acid extraction/purification, amplification, and detection. All stages have a major bearing on the quality and reliability of laboratory testing results. For a NAAT to successfully diagnose STIs, a clinical specimen needs to be properly sampled from the appropriate anatomic site, and the nucleic acids of targets stabilized in the transport medium for storage and transfer. In the laboratory, efficient nucleic acid extraction/purification is crucial for sample preparation to produce a pure PCR template that is free of contaminating nucleases and inhibitors. Subsequently, a well-designed and optimized NAAT is essential for the efficient amplification to produce sufficient PCR

amplicons; last but not least, a proper detection strategy needs to be in place to produce clear and strong signals for positivity, and evidently negative signals for negativity. The process of NAAT testing is considered to be medium to high complexity, and generally requires well-trained personnel and sophisticated laboratory settings.

The high sensitivity of NAATs can be problematic because even a very low level of contamination can lead to false positives. The possibility of contamination, which is the biggest concern for many molecular diagnostic laboratories, can come from almost all stages of the nucleic acid-based processing. In some methods, the addition of enzyme such as Uracil N-glycosylase to the PCR master mix preparation helps to reduce amplicon carry-over contamination by degrading the amplicons, but it does not prevent genomic DNA contamination directly from specimen to specimen. Thus, good molecular laboratory practices, uni-directional work flow, separate rooms for specimen nucleic acid extraction, PCR master mix preparation, and testing are pre-requisites for a successful NAAT. Another alternative to reduce the possibility of contamination is to deploy a closed and robotic system as in many commercial platforms for STI testing. Other than the sample collection in the corresponding proprietary transport medium, the remaining stages of the NAAT process is fully automated, eliminating the labour-intensive sample processing steps. However, the molecular test complexity, the need for sophisticated and often expensive equipment, costly NAAT test kits, as well as the need for experienced technicians can provide a challenge for laboratories with limited resources.

At the time of writing this annex (June 2012), two major categories of diagnostic technologies exist for the identification of STIs using molecular methods. One category (amplified nucleic acid technologies; i.e. NAATs), which is the most widely used, is based on amplification of specific nucleic acid (DNA or RNA) from the target microorganism to generate sufficient amplified products to be converted through different technologies to signals for detection. The second category (non-amplified nucleic acid-based technology) uses non-amplified, nucleic acid probes (e.g. labelled with enzyme) that hybridize directly to the target template and subsequently

Table A3.1: Examples of commercially available nucleic acid-based assays for STIs (June 2012)

Target organisms	Technology	Company
Chlamydia trachomatis (CT), *Neisseria gonorrhoeae* (NG), or CT/NG combo	PCR TMA-HPA HC (DNA probe) Real-time PCR SDA HC (RNA probe)	Roche, Cepheid Gen-Probe Gen-Probe Abbott, Roche, Siemens Becton, Dickinson Qiagen
HIV	RT-PCR Real-time PCR Real-time PCR NASBA Real-time PCR RT-PCR bDNA	Roche Abbott bioMérieux National Genetics Institute Siemens
HIV genotyping	Sequencing Sequencing	Siemens Abbott
HPV	Real-time PCR TMA-HPA HC PCR	Abbott Gen-Probe Qiagen Roche
HPV genotyping	PCR PCR-line hybridization	Roche Innogenetics
HBV	Real-time PCR Real-time PCR bDNA HC	Roche Abbott Siemens Qiagen
HBV genotyping	Sequencing	Siemens
HCV	Real-time PCR RT-PCR Real-time PCR RT-PCR bDNA	Roche Abbott National Genetics Institute Siemens
HCV genotyping	RT-PCR	Abbott
HSV	Real-time PCR Real-time PCR Real-time PCR Real-time PCR SDA Helicase-dependent isothermal amplification	Abbott Roche Qiagen EraGen Biosciences Becton, Dickinson BioHelix

Table A3.1: Examples of commercially available nucleic acid-based assays for STIs (June 2012) (*continued*)

Target organisms	Technology	Company
Trichomonas vaginalis	TMA-HPA	Gen-Probe
C. trachomatis, N. gonorrhoeae, and *Mycoplasma genitalium*	PCR	Bio-Rad Laboratories
Gardnerella vaginalis, T. vaginalis, and *Candida spp.*	DNA probe hybridization	Becton, Dickinson

bDNA, branched chain DNA hybridization assay; HBV, hepatitis B virus; HC, hybrid capture; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPA, hybridization protection assay; HPV, human papillomavirus; HSV, herpes simplex virus; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SDA, strand displacement amplification; TMA, transcription-mediated amplification.

through, e.g. enzyme-substrate reactions, the signals are amplified and detected. Non-amplified nucleic acid-based technologies usually are less expensive, and the analytical sensitivity exceeds that of conventional diagnostic methods such as culture or enzyme immunoassays (EIAs), but the sensitivity is substantially lower than that of the amplified technologies. Table A3.1 lists some of the commercially available nucleic acid-based assays for the molecular diagnosis of STIs at the time of this writing.

A3.2 Amplified nucleic acid technologies

NAATs are the most sensitive yet complex among the molecular diagnostic tests and generally have superior performance in terms of sensitivity over the direct (non-amplified) probe-based tests as well as all other classes of diagnostic test. Some of the first-generation commercial NAATs for the diagnosis of STIs have been partially automated to reduce labor requirements, but are still at risk of contamination problems. Currently, second-generation commercial testing platforms on the market are equipped with improved specimen-processing modules and fully automated to achieve highly efficient and contamination-free molecular diagnostics and large-scale screening. Commercial amplification-based molecular diagnostic systems have focused mainly on assays for detection of *N. gonorrhoeae, C. trachomatis*, herpes simplex virus (HSV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and human papillomavirus (HPV). In addition to qualitative detection of viruses, quantification of viral load in clinical specimens is of great value and available for diagnosis, prognosis, and therapeutic monitoring for HIV, HBV, and HCV. Table A3.2 lists some commercial

nucleic acid amplification technologies, showing the companies that market these tests, whether or not the tests are isothermal, the type of target, and the enzymes they use. As mentioned above, one of the main strengths of these highly sensitive amplified assays is their capability of testing noninvasive sample types, such as first-catch urine samples and vaginal swabs, that can be self-obtained. The successful use of multiplex amplified assays and noninvasive sample types has greatly facilitated the screening process, which is particularly important since most STIs are asymptomatic and their detection depends on screening populations that ordinarily would not be tested by traditional approaches. It is now possible to use alternative venues, including home-based specimen collection for screening programmes outside STI or family planning clinics, where a clinician-collected specimen is not required. First-catch urines from men and self-obtained vaginal swabs from women are highly acceptable for *C. trachomatis* and *N. gonorrhoeae* screening programmes when they are used with amplified nucleic acid technologies. This noninvasive means of obtaining a diagnostic specimen may provide a way to reach sexually active individuals who should be screened for STIs but lack adequate access to health care or fear pelvic examinations. It also can be cost saving when expensive pelvic examinations performed by the clinician are not required.

A3.2.1 Polymerase chain reaction (PCR)

PCR was developed in 1983 (*5*, *6*) and often is heralded as one of the most important scientific advances in biology and medicine. It is now a fundamental and often indispensable technique widely used in molecular

Table A3.2: Nucleic acid amplification tests (NAATs), June 2012

Technology	Company	Isothermal	Target	Enzymes
PCR	Roche	No	DNA	DNA polymerase
Real-time PCR	Abbott, Roche	No	DNA	DNA polymerase
TMA	Gen-Probe	Yes	RNA	Reverse transcriptase and RNA polymerase
SDA	Becton, Dickinson	Yes	DNA	DNA polymerase and restriction endonuclease
NASBA	bioMérieux	Yes	RNA	Reverse transcriptase, RNase H, RNA polymerase

NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; SDA, strand displacement amplification; TMA, transcription-mediated amplification.

biology, microbiology, clinical diagnostics, and many other applications. The name, PCR, comes from the DNA polymerase used to amplify a specific sequence of DNA by in vitro enzymatic replication. The initial heating step (usually at 94–95°C) denatures the original target double-stranded DNA (dsDNA) into two complementary single strands of DNA, allowing two primers to anneal at a lower temperature (dependent on primers) to their complementary sequences, flanking the specific sequence to be amplified. Next, the extension (usually at 72°C) of the primers is catalysed by a heat-stable DNA polymerase (such as *Taq* DNA Polymerase), which synthesizes two new strands of DNA, using deoxyribonucleoside triphosphates (dNTPs) and the original strands as templates. This process (Fig. A3.1) results in the duplication of the original target DNA sequence, with each of the new molecules containing one old and one new strand of DNA. Subsequently, each of these strands can be used to create two new copies, and so on. Numerous repetitive cycles of denaturing, annealing, and extension can result in exponential accumulation of a specific DNA fragment. The three-step PCR cycles can be repeated as many as 30 or 40 times, leading to billions of copies of the original DNA target sequence. The entire cycling process of PCR is automated, programmable, and can be completed in just
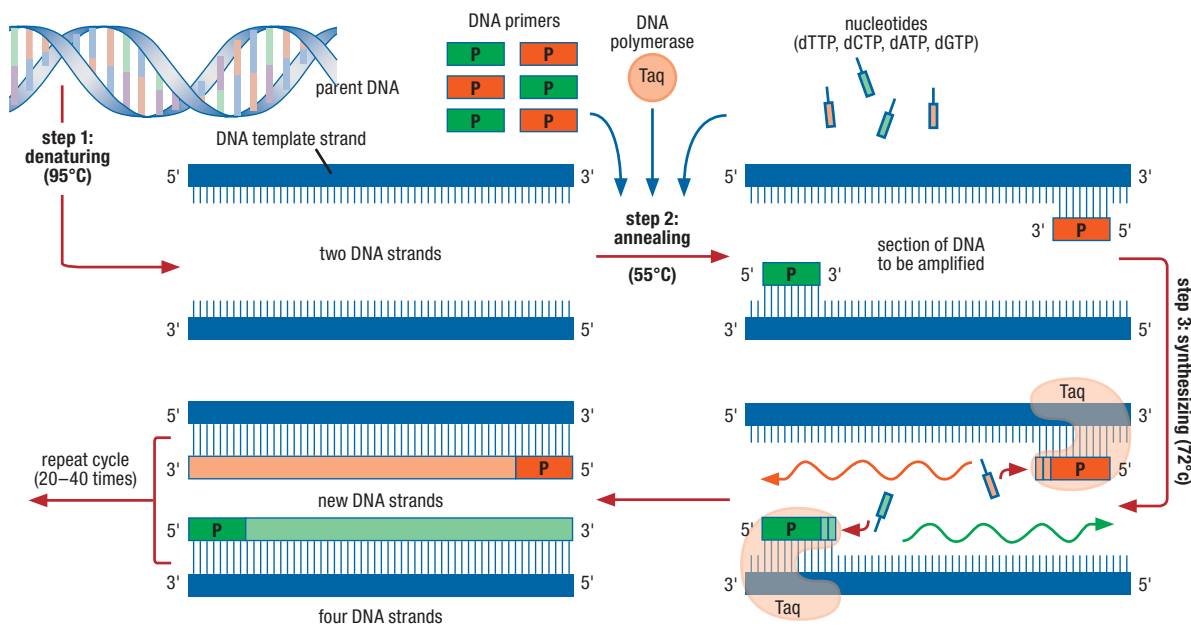

Figure A3.1
Polymerase chain reaction (PCR): three-step process
Source: Courtesy of the Encyclopædia Britannica, Inc. (http://www.britannica.com/EBchecked/media/18071/The-three-step-process-of-the-polymerase-chain-reaction, accessed 23 June 2013).

a few hours. It is performed in a machine called thermocycler, which is equipped with elements and/or fans for heating and cooling to rapidly alter the temperature to allow DNA denaturation, primer annealing, and extension. It is important to note that appropriate maintenance and verification of temperatures and ramp times of thermocyclers are keys to ensure high-quality, reproducible results.

PCR technology has revolutionized the detection and characterization of infectious disease organisms, including the etiological agents of STIs. Traditionally, specific amplicons generated by PCR were detected by ethidium bromide-stained gel electrophoresis. Other amplicon detection methods include the use of capture and detection probes (labelled with, e.g. enzymes or fluorescent molecules) in a microwell format, and the sizing of PCR products using a Genetic Analyzer (Applied Biosystems) or Bioanalyzer (Agilent). Many modifications and adaptations have been made to the original PCR technique to expand its usage and benefits, which include, but are not limited, to the following techniques: nested PCR, touchdown PCR, hot-start PCR, reverse transcriptase (RT) PCR (for RNA target), asymmetric PCR, digital PCR, multiplex PCR, semiquantitative PCR, and quantitative real-time PCR.

A3.2.2 Real-time PCR

The principle of real-time PCR is identical to that of conventional PCR except that the amplification and detection processes are combined to allow monitoring the PCR amplification as it occurs in real time; conventional PCR is capable only of end-point measurement (i.e. to detect or visualize PCR products after amplification has been completed). The most significant advance of a real-time PCR design is the incorporation of fluorescence-based detection technology using either dsDNA-binding dyes, or specific fluorescent molecule-labelled probes (such as hydrolysis, hybridization, or conformational probes, molecular beacons, for example) to allow not just the detection of the specific nucleic acid target but also quantification of the target (Q-PCR). In addition, real-time PCR integrates and automates both amplification and detection in an instrument, which often requires less time and labour for results, compared to performing the conventional PCR. Other advantages of real-time PCR include the use of shorter target sequences (<150 bp), less specimen volume required, broader

dynamic range of detection, higher analytical sensitivity and specificity, capability of multiplexing and melt-curve analysis, and that no-post PCR manipulation is required.

To monitor amplification during real-time PCR, fluorescent reporter molecules are used for the generation of fluorescence signals. Most real-time PCR systems use a fixed array of light-emitting device for fluorescence excitation and a charge-coupled device camera or photomultiplier tube with proper filters for fluorescence detection. The increase in fluorescent signal is directly proportional to the number of PCR amplicons generated in the exponential phase of the reaction. The baseline of a real-time PCR refers to the level of signal during the initial 5–15 cycles in which there is little change in fluorescent signal, whereas the threshold is the level of signal that reflects a significant increase over the calculated baseline signal. The threshold can be set automatically by the instrument software or manually by the user. The cycle threshold (Ct) is the PCR cycle number where the fluorescent signal of the reaction crosses the threshold. Accordingly, the presence of a Ct reflects the accumulation of a sufficient number of amplicons to be considered as a positive reaction, and the Ct is inversely related to the amount of starting template. The real-time PCR data are usually plotted on a graph as PCR cycle number versus the intensity of fluorescence, which is related to the number of starting template. Figure A3.2 shows a typical real-time PCR amplification curve.
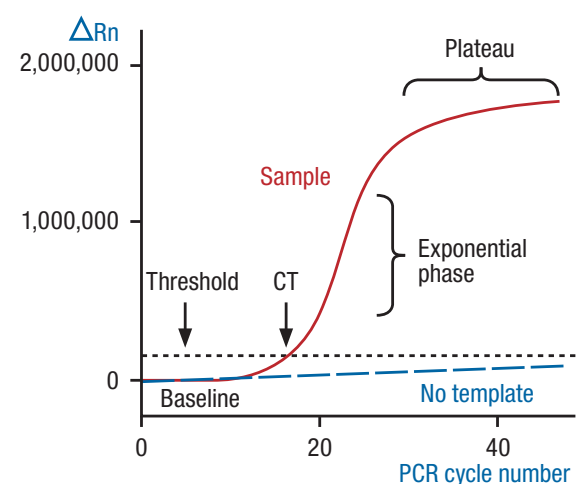
Figure A3.2
Typical real-time PCR amplification curve
Source: Courtesy of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml, accessed 23 June 2013).

Two common methods for the detection of amplification products in real-time PCR are: non-specific detection using fluorescent dyes that intercalate with any dsDNA, such as SYBR Green; and specific detection using target-specific fluorescent probes, such as TaqMan probe (hydrolysis probe), molecular beacons, or dual hybridization probes. SYBR Green is probably the most widely used dsDNA-binding dye for real-time PCR. In the solution, the unbound dye molecules exhibit very little fluorescence, but the fluorescence increases significantly when bound to dsDNA. As dsDNA accumulates, SYBR Green generates signals that are proportional to the concentration of the target DNA (Fig. A3.3A). This technology is considered to lack very high specificity because the dye binds indiscriminately to all dsDNA formed during PCR and, consequently, any spurious amplification products may also contribute to the overall fluorescent signals. However, amplification that uses dsDNA-binding dyes can be subjected to melting curve analysis to differentiate between specific and non-specific PCR products using melting peaks (Fig. A3.3B).

(A)

(B)
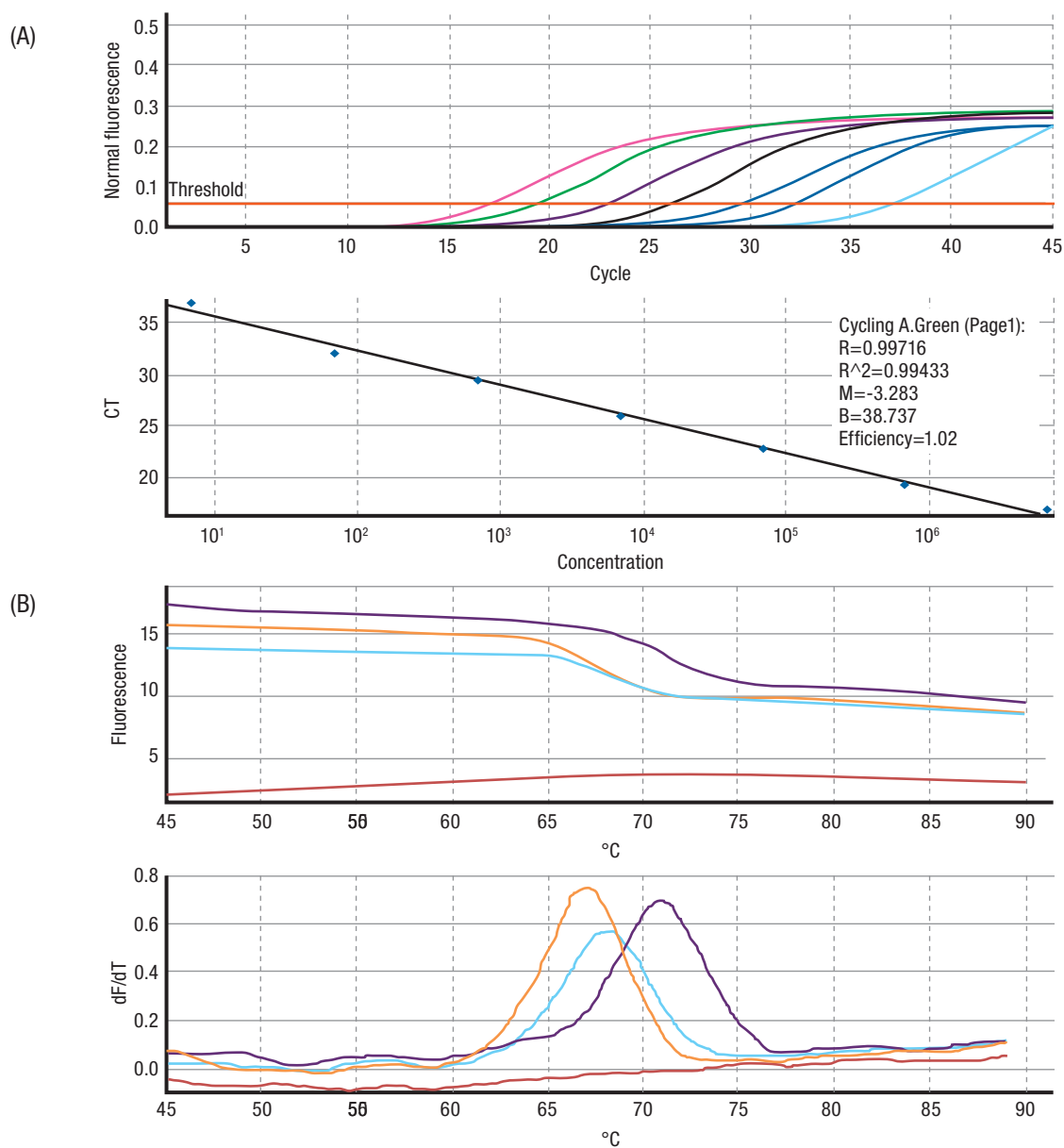
Figure A3.3
(A) Typical real-time PCR amplification curves showing specimens with a serial dilution of target DNA and the standard curve; (B) Melting curve analysis using melting temperatures (Tm) to enhance the specificity of SYBR Green-based real-time PCR.
Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

TaqMan probe (hydrolysis probe; Fig. A3.4) is the most commonly used target specific probe in the real-time PCR detection. The TaqMan probe-based PCR requires a pair of specific PCR primers and an oligonucleotide TaqMan probe complementary to a specific DNA sequence of the template between the forward and reverse primers. The probe usually is designed with a high-energy dye termed Reporter (fluorophore) at the 5' end, and a low energy molecule named Quencher at the 3' end (that quenches the fluorescence of the reporter). This dually labelled fluorescent probe emits little fluorescence when free in solution, due to the close proximity of the quencher to the reporter. During the real-time PCR amplification, the probe annealed to the template is cleaved by the 5' nuclease activity of the Taq DNA polymerase to release (separate from the quencher); upon excitation by a light source, the reporter dye generates fluorescence that increases for each PCR cycle. TaqMan probe-based assays have been used widely in the real-time PCR for gene expression, viral load determination, single nucleotide polymorphism (SNP) genotyping, bacterial identification, allelic discrimination, and verification of microarray results.

Molecular beacons (hairpin probes) are single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure (Fig. A3.5). The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of the complementary arm sequences that are located on both sides of the probe sequence. A fluorophore is covalently linked to the 5' end of one arm and a quencher is covalently linked to the 3' end of the other arm. The stem keeps these two molecules in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer, thus molecular beacons do not fluoresce when they are free in solution. However, when they hybridize to a nucleic acid strand containing its complementary target sequence they undergo a conformational change that enables them to fluoresce brightly. Accordingly, when the probe hybridizes to a target molecule, it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid. Consequently, the molecular beacon undergoes a spontaneous

TaqMan probe-based assay chemistry

1. **Polymerization:** A flurescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe, respectively
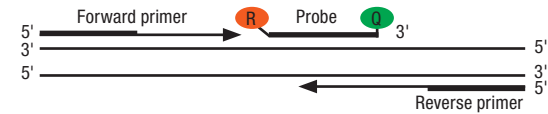
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.
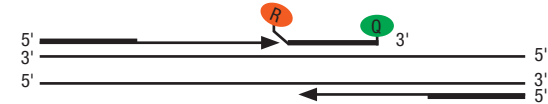
3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.
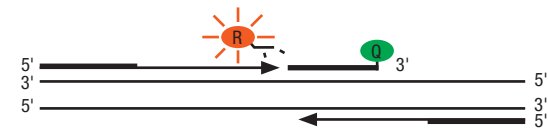
4. **Polymerization completed:** Once seperated from the quencher, the reporter dye emits its characteristic fluorescence.
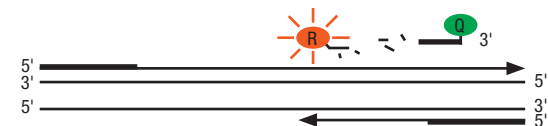
Figure A3.4
TaqMan probe (hydrolysis probe)-based real-time PCR
Source: Courtesy of the Life Technologies Corporation (http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html, accessed 23 June 2013).

Figure A3.5
Molecular beacon (hairpin probe)
Source: Courtesy of Horspool D. Wikipedia, 2009 (http://en.wikipedia.org/wiki/Molecular_beacon, accessed 11 April 2013); *Introduction to molecular beacons*. New Jersey, USA, Public Health Research Institute, 2013 (http://molecular-beacons.org/MB_introduction.html, accessed 23 June 2013).

conformational reorganization that forces the stem apart and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. Molecular beacons can be used in the real-time PCR assay for SNP detection and mutation detection.

The dual hybridization or fluorescence resonance energy transfer (FRET) probe system (Fig. A3.6) consists of two partnering fluorescent probes that hybridize on the target sequence in close proximity (usually one to a maximum of four nucleotides apart). The donor (anchor) probe is labelled with a fluorophore at the 3' end and the acceptor (reporter) probe with another fluorophore at the 5' end. The fluorophores are designed to allow the emission spectrum of one to overlap significantly with the excitation spectrum of the other. During PCR, the donor fluorophore is excited by an external light source, and the energy is transferred to the acceptor fluorophore if positioned adjacent to the former. The excited acceptor fluorophore then emits light at a different wavelength, which then can be detected and measured. FRET probes often are used in real-time PCR for, e.g. allelic discrimination assays.

A3.2.3 Transcription-mediated amplification (TMA)

The patented TMA technology (7, 8) can be used to amplify either DNA or RNA, and produces RNA amplicon in contrast to most other NAATs that only produce DNA. In available commercial TMA systems, a target capture (TC) process is performed before TMA, i.e. to reduce inhibition of amplification and contamination. In this TC method, poly-T oligomers bound to magnetic particles are used to bind a capture probe containing poly-A tail and target-specific sequence, which then can specifically hybridize to the complementary sequence of the target RNA. The subsequent TMA process (Fig. A3.7), which amplifies the captured target sequence, uses two target-specific primers and two different enzymes (RT and RNA polymerase) for amplification. One of the target-specific primers contains a promoter sequence for the RNA polymerase and when this primer hybridizes to the target RNA, a reverse transcription is initiated, creating a complementary DNA (cDNA) copy. The RNA in the resulting RNA:DNA heteroduplex is degraded by the RNase H activities of the RT. This enables the second primer to bind to the DNA copy and synthesize a new strand of DNA by the RT, creating a dsDNA molecule. Both strands of the created dsDNA molecule now contain promoter sequences for RNA polymerase, and, therefore, can be used as a template to initiate transcription. Subsequently, each of the newly synthesized RNA amplicons reenters the TMA process and serves as a template for a new round of replication, leading to an exponential expansion of the RNA target sequence. TMA can produce 100–1000 of copies per amplification cycle in contrast to PCR that produces only 2 copies per cycle. TMA is isothermal; the entire reaction is performed at the same temperature in a water-bath or heat block instead of a thermal cycler.

3' donor fluorophore (F_D)

Oligo probe 1
5' acceptor fluorophore (F_A)
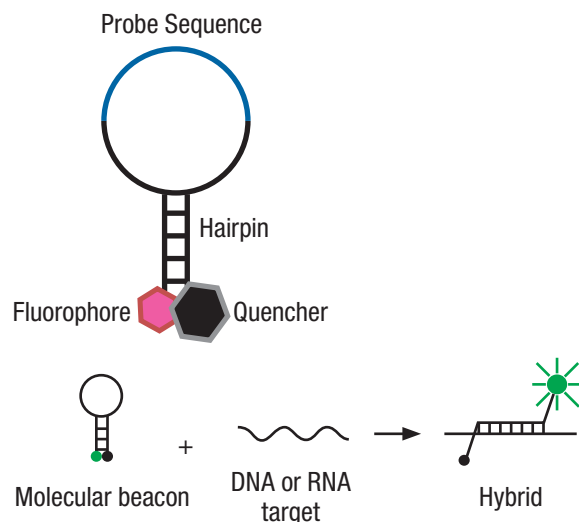
Oligo probe 2

1. Probes in solution emit low fluorescence

Amplified target DNA

2. Emission through fluorescence resonance energy transfer
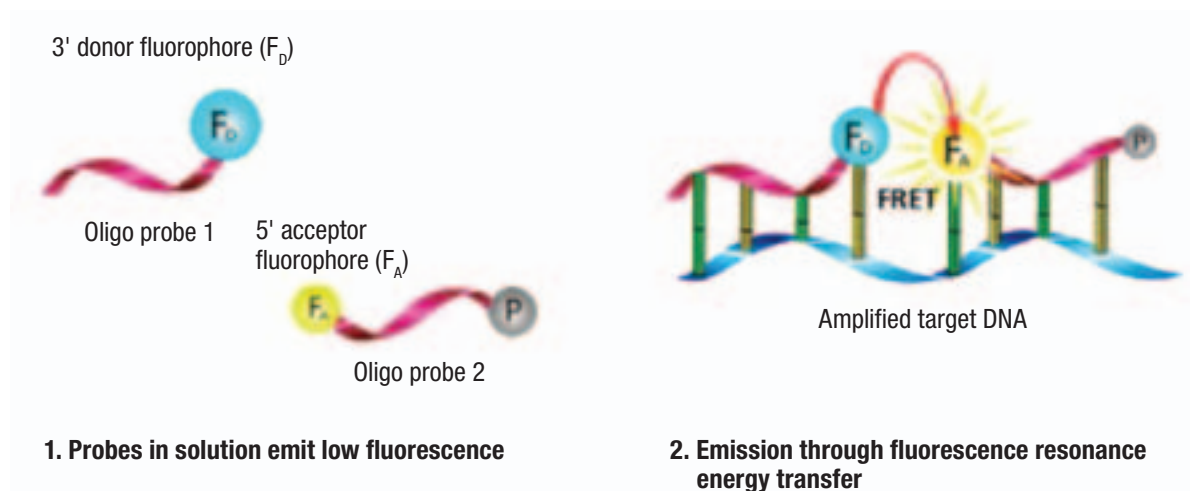
Figure A3.6
Dual hybridization or fluorescence resonance energy transfer (FRET) probe system
Source: Courtesy of SIGMA-ALDRICH, St Louis, MO, USA (http://www.sigmaaldrich.com/life-science/custom-oligos/dna-probes/product-lines/fluorescent-probes/lightcycler-probes.html, accessed 23 June 2013).
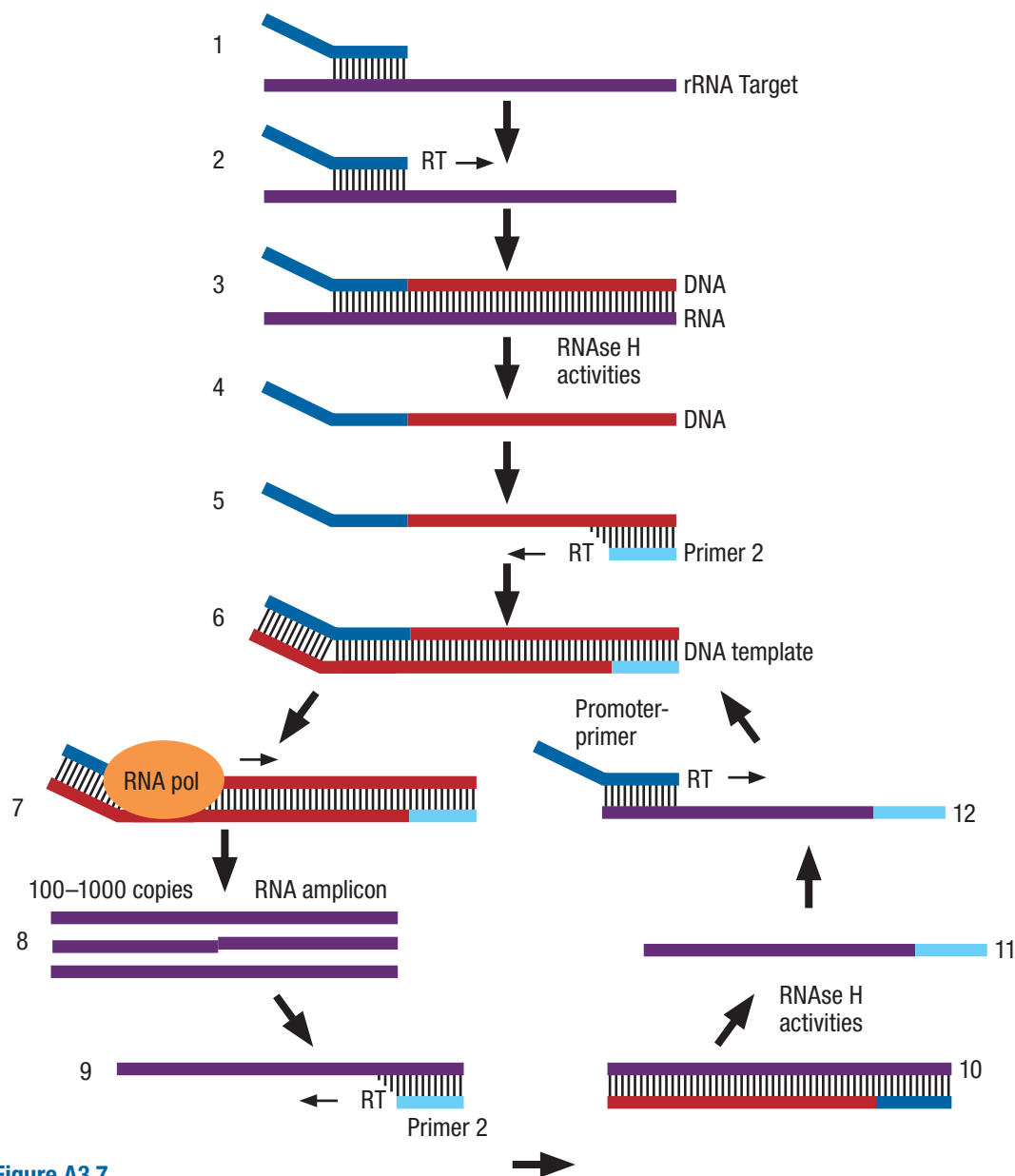
Figure A3.7
Transcription-mediated amplification (TMA)
Source: Hill CS. *Gen-Probe transcription-mediated amplification: system principles.* Courtesy of Gen-Probe Inc., San Diego, CA, USA (http://www.gen-probe.com/pdfs/tma_whiteppr.pdf, accessed 23 June 2013).

The RNA amplicons produced in the TMA reaction can be combined with molecular torches for real-time detection or with a specific gene probe in hybridization protection assay (HPA) for chemiluminescence end-point detection. The HPA technique is described under non-amplified nucleic acid-based technologies (see section A3.3.2).

A3.2.4 Nucleic acid sequence-based amplification (NASBA)

NASBA (*8*, *9*) is another isothermal amplification technology for RNA or DNA target sequences. The technology is similar to TMA except that NASBA uses

three (instead of two) enzymes: Avian Myoblastosis Virus-RT (AMV-RT), RNase H, and T7 RNA polymerase. These enzymes together with specific primers enable the amplification of the target nucleic acid sequence (Fig. A3.8). Amplified RNA products can be detected by post-amplification hybridization using electrochemiluminescently labelled (ECL) probes or by a real-time detection system using molecular beacons.

A3.2.5 Strand displacement amplification (SDA)

SDA is an additional isothermal, in vitro enzymatic process that permits the amplification of target
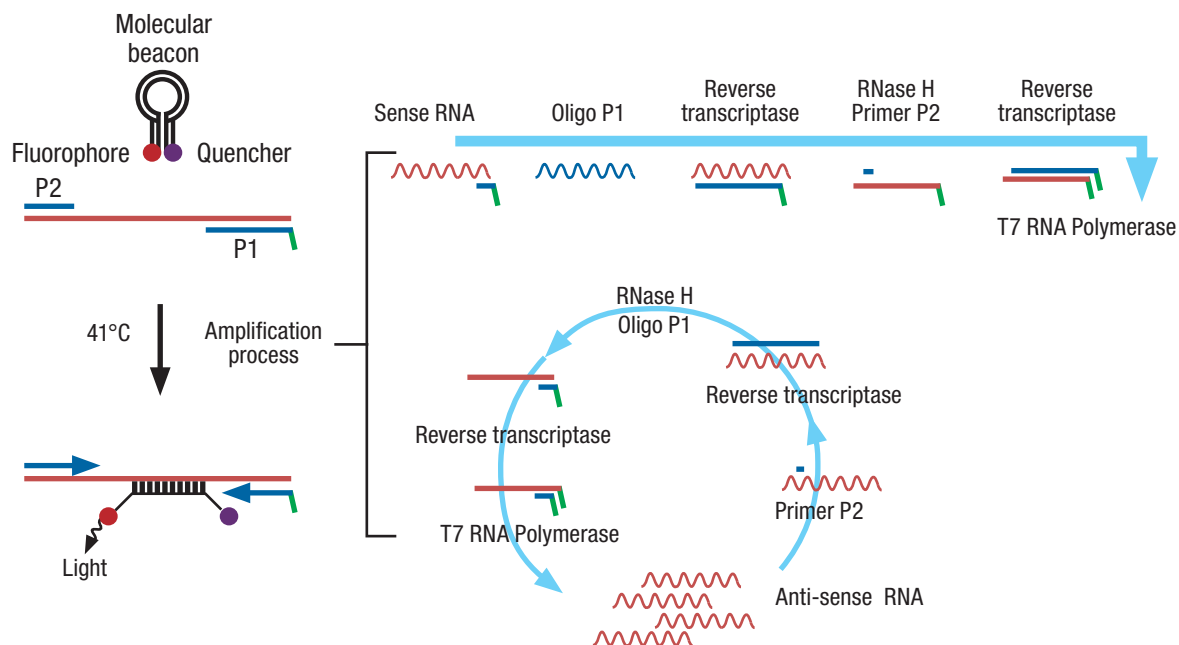
Figure A3.8
Nucleic acid sequence-based amplification (NASBA)
Source: *NASBA technology.* Courtesy of PREMIER Biosoft, Palo Alto, CA, USA (http://www.premierbiosoft.com/tech_notes/NASBA.html, accessed 23 June 2013).

molecules from a single DNA or RNA template (*8, 10*). The patented technology is based on the combined action of a restriction enzyme, DNA polymerase, and two pairs of primers (SDA amplification primers and Bumper [Adapter] primers). The Bumper primers contain only target-specific DNA sequences, while the SDA amplification primer pairs contain specific restriction endonuclease recognition sequence at its 5' end in addition to a sequence complementary with the target segment. SDA consists of two phases. Figure A3.9 shows the SDA process. The first phase is the generation of dsDNA of the template of interest containing restriction endonuclease recognition site that feeds into the second phase of exponential amplification where the restriction endonuclease nicks one of the two strands of the newly formed dsDNA, allowing the DNA polymerase to create a new dsDNA sequence from the displaced strand. This process, which resembles the rolling-circle replication of single-stranded phages and small plasmids, is repeated continuously until a sufficiently large amount of the DNA strand of interest is produced and can be detected by a detector probe.

Currently in a second-generation commercial platform, the real-time detection of SDA products occurs simultaneously with amplification using fluorescent detector probe and fluorescence energy transfer (Fig. A3.10). The detector probe consists of a target-specific hybridization region at the 3' end and a hairpin structure at the 5' end. The loop of the hairpin contains the restriction endonuclease recognition sequence, and the 5' base is conjugated to the donor molecule, while the 3' base of the hairpin stem is conjugated to an acceptor molecule. In its native state, the hairpin maintains the donor and the acceptor molecules in close proximity and little fluorescence is observed. As the detector probe anneals to the target, the hairpin becomes linearized and extended by the polymerase. This extension creates a double-stranded detector probe with a cleavable restriction site, which the restriction enzyme promptly cleaves. The cleavage causes the physical separation of the donor from the quenching effects of the acceptor and allows real-time detection of fluorescence.

A3.3 Non-amplified nucleic acid-based technologies

There are also non-amplified nucleic acid-based tests available for certain STIs, such as gonorrhoea, chlamydia, hepatitis, and HIV. They usually are rapid, can be automated for large scale screening, are relatively inexpensive, and only require moderate technical skill. However, their

A. Target generation

SDA Amplification Primer (AP) and Bumper Primer (BP) hybridize to target DNA strand (TS).

DNA Polymerase

SDA Amplification Primer (AP) and Bumper Primer (BP) are extended by DNA Polymerase along target strand.

Extension and Displacement

Extension of Bumper Primer displaces Amplification Primer extension product.

Hybridization of Complementary SDA Primer and Exponential Amplification

B. Exponential amplification

Hybridization of SDA Amplification Primer (AP1) and Detector Probe (DP) to target DNA Sequence (TS2).

Extension of Amplification Primer displaces Detector Probe extension product (conversion of Detector Probe shown below) forming duplex with restriction enzyme (BsoBI) site.

Restriction enzyme nicks at restriction site; DNA polymerase binds to nick and extends to displace strand. DNA strands that are displaced into solution are captured by complementary primers that undergo the same process of extension followed by nicking and displacement, thereby resulting in exponential amplification as depicted.

Figure A3.9
Strand-displacement amplification (SDA)
Source: Courtesy of Becton, Dickinson and Company, Franklin Lakes, NJ, USA.

sensitivity is considerably less than that of target amplification-based (NAATs) methods. Most direct probe hybridization and detection assays are more likely to be used when large amounts of target DNA or RNA are expected to be present, for example, a urethral swab or a bacterial culture. A reliable detection of target without the use of signal amplification usually would require at least 10^4 copies of nucleic acid target per microlitre. However, amplification of the signal after probe hybridization improves detection to as low as approximately 500 target molecules per microlitre and provides quantitative capabilities.

A3.3.1 Hybrid capture (HC)

HC technology is an in vitro nucleic acid hybridization assay with signal-amplification using microplate chemiluminescence for the qualitative detection of nucleic acid targets (11). Basic steps of the HC assay involve the lysis of the virus or bacteria to release target DNA or RNA, hybridization of specific RNA or DNA probes to create RNA:DNA hybrids, capture of RNA:DNA hybrids onto a solid phase using universal antibodies specific for the hybrids, amplification of signal with antibodies conjugated to an enzyme (e.g. alkaline phosphatase), detection of

Detector Probe Conversion

Hybridization of SDA Amplification Primer (AP1) and Detector Probe (DP) to target DNA Sequence (TS2).

Extension of Amplification Primer displaces the Detector Probe extension product.

Detector Probe extension product becomes target for SDA Amplification Primer (AP2).

Amplification Primer and Detector Probe extension product are both extended in the 3' direction forming duplex.

The restriction site on the Detector Probe is different from that incorporated in the Amplification Primer sequences and remains susceptible to cleavage by BsoBI. Thus, fluorophore and quencher dyes diffuse apart, resulting in increased fuorescence. In the absence of target DNA, Detector Probes remain uncut and fluorescence continues to be quenched.

Figure A3.10
Second-generation strand-displacement amplification (SDA), in which amplified products are detected in real time using fluorescent detector probe and energy transfer.
Source: Courtesy of Becton, Dickinson and Company, Franklin Lakes, NJ, USA.

signal when substrate (e.g. chemiluminescent dioxetane) is cleaved by the enzyme, and measurement of chemiluminescence produced in relative light units (RLUs) using a luminometer (Fig. A3.11).

A3.3.2 Hybridization protection assay (HPA)

HPA technology is a patented method that involves the detection of RNA or single-stranded DNA targets by means of a chemiluminescent DNA probe (12, 13). In the HPA process, sequence-specific DNA probes labelled with acridinium ester (AE) are allowed to hybridize to the amplification products. Separation (selection) of hybridized from unhybridized probes is done by the addition of selection reagent (alkali), which hydrolyses the AE label on unhybridized probes. When the probe binds to its specific target sequence, the AE label on the hybridized probe is protected within the double helix and is not hydrolysed. Upon the addition of detection reagent, only the AE label attached to the hybridized probe is left to produce a signal indicating that the target DNA or RNA is present. No chemiluminescence is emitted from the

unhybridized probes (Fig. A3.12). HPA does not require the cumbersome wash steps needed with conventional probe tests and immunoassays. Furthermore, only one molecule of AE-labelled probe can bind to each RNA amplicon; thus, the chemiluminescent signal obtained is directly proportional to the number of target molecules in the initial sample. The dual kinetic assay (DKA) technology, which is a modification of the HPA technology, uses two types of probes labelled with two different AEs displaying different light-off kinetics in a single assay that enables the detection of two separate targets simultaneously. HPA or DKA technology is commonly used for the detection of RNA amplicons produced by TMA.

A3.3.3 Branched-chain DNA hybridization (bDNA)

In contrast to PCR technology that relies on in vitro amplification of the target sequence, bDNA involves the use of a series of oligonucleotides in a sandwich nucleic acid hybridization method to detect and quantify target by signal amplification (14). The process of bDNA involves the lysis of target organisms, target capture, signal

1. **Hybridize RNA probe with target DNA.** Target DNA combines with specific RNA probes, creating RNA:DNA hybrids.

2. **Hybrid capture.** RNA:DNA hybrids are captured onto a solid phase coated with universal capture antibodies specific for RNA:DNA hybrids.

3. **Signal amplification.** Capture RNA:DNA hybrids are detected with multiple antibodies conjugated to alkaline phosphatase. The signal resulting from the chemiluminescent reaction is read and results interpreted.

Figure A3.11
Hybrid capture (HC) technology
Source: Courtesy of Qiagen Inc., Germantown, MD, USA (http://www.qiagen.com/hpv/hc2technology.aspx).
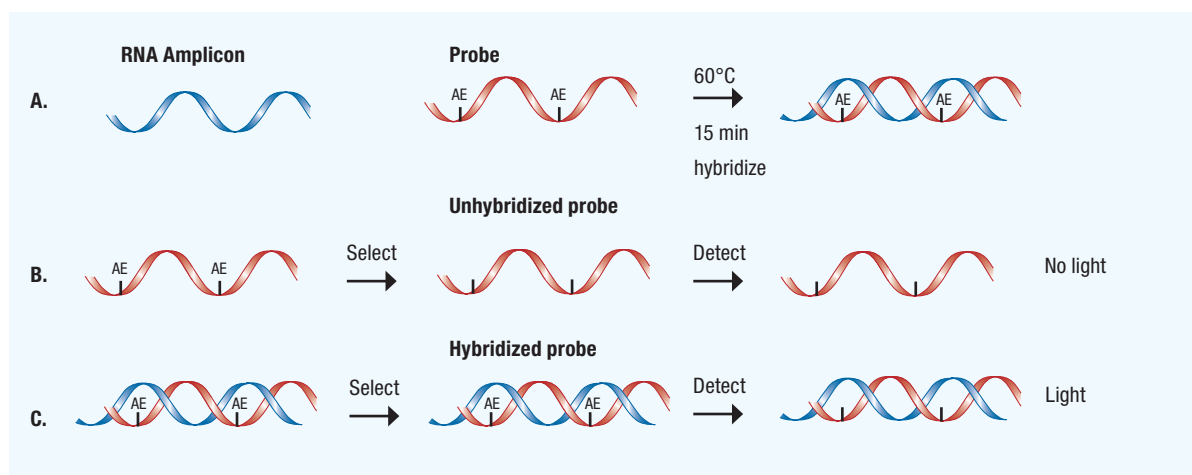
Figure A3.12
Hybridization protection assay (HPA)
Source: Hill CS. *Gen-Probe transcription-mediated amplification: system principles*. Courtesy of Gen-Probe Inc., San Diego, CA, USA (http://www.gen-probe.com/pdfs/tma_whiteppr.pdf, accessed 23 June 2013).

amplification, and detection, as shown in Figure A3.13. The initial step of a bDNA assay is to disrupt the target organisms using detergent and proteinase K to release nucleic acids (A). The first set of target-specific oligonucleotides (capture extender) then are hybridized with high stringency to both the target nucleic acids as well as the capture probes that are attached to a microwell plate (B). The second set of oligonucleotides (label extenders) is designed to hybridize to contiguous regions on the target and to provide sequences for hybridization of a preamplifier oligonucleotide. The preamplifier forms a stable hybrid only if it hybridizes to two adjacent label extenders (C). Multiple bDNA amplifier molecules then are hybridized to the preamplifier to create a branched structure (D). Finally, alkaline phosphatase-labelled oligonucleotides that are complementary to bDNA amplifier sequences bind to the bDNA molecule by hybridization. The bDNA signal is the chemiluminescent product from the alkaline phosphatase and its specific substrate. In this way, the signal is amplified without copying the target nucleic acid sequence, and the amount of signal detected is directly proportional to the amount of bound nucleic acid.

bDNA technology has progressed from the first-generation assays that were accurate and reproducible but relatively insensitive, to third-generation tests that are accurate, reproducible, highly sensitive, and amenable to full automation. In addition, bDNA assays do not require the amplification of a target sequence, thus cross-contamination between replicate samples or carryover is less likely in bDNA assays.
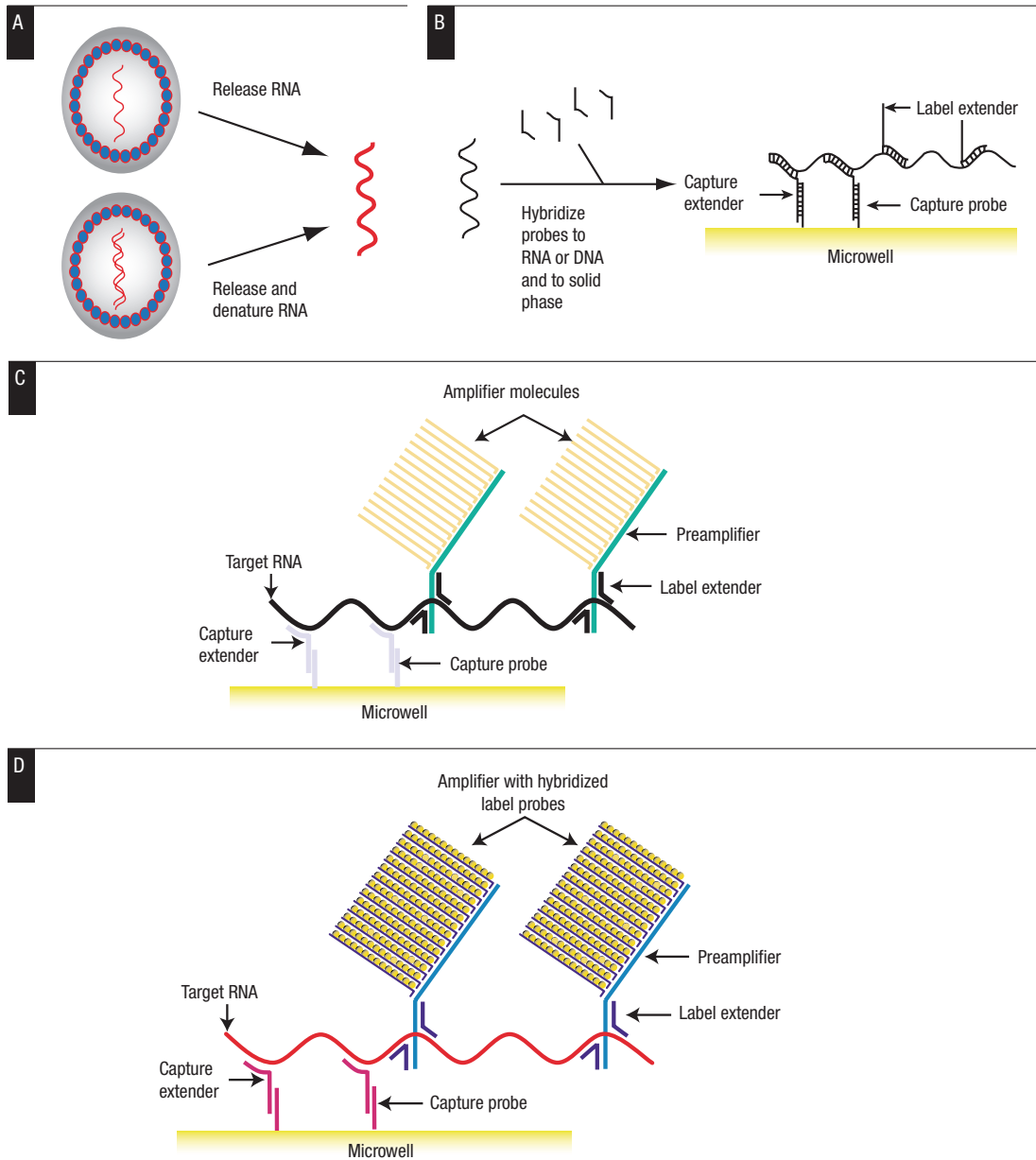
Figure A3.13

Branched chain DNA (bDNA) signal amplification assay

Source: Tsongalis, GJ. Branched DNA technology in molecular diagnostics. *American Journal of Clinical Pathology*, 2006, 126:448–453.

A3.4 References

1. Palmer HM et al. Evaluation of the specificities of five DNA amplification methods for the detection of *Neisseria gonorrhoeae*. *Journal of Clinical Microbiology*, 2003, 41(2):835–837.

2. Tabrizi SN et al. Evaluation of six commercial nucleic acid amplification tests for the detection of *Neisseria gonorrhoeae* and other Neisseria species. *Journal of Clinical Microbiology*, 2011, 49(10):3610–3615.

3. Ripa T, Nilsson P. A variant of *Chlamydia trachomatis* with deletion in cryptic plasmid: implications for use of PCR diagnostic tests. *Eurosurveillance*, 2006, 11(11):E061109.2.

4. Unemo M, Clarke IN. The Swedish new variant of *Chlamydia trachomatis*. *Current Opinion in Infectious Diseases*, 2011, 24(1):62–69.

5. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, 1987, 155:335–350.

6. Saiki RK et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 1988, 239(4839):487–491.

7. La Rocco MT et al. Evaluation of a commercial rRNA amplification assay for direct detection of *Mycobacterium tuberculosis* in processed sputum. *European Journal of Clinical Microbiology and Infectious Diseases*, 1994, 13(9):726–731.

8. Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies: a review. *Nucleosides, Nucleotides and Nucleic Acids*, 2008, 27(3):224–243.

9. Compton J. Nucleic acid sequence-based amplification. *Nature*, 1991, 350(6313):91–92.

10. Little MC et al. Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. *Clinical Chemistry*, 1999, 45(6):777–784.

11. Vernick JP, Steigman CK. The HPV DNA virus hybrid capture assay: what is it—and where do we go from here? *Medical Laboratory Observer*, 2003, 35(3):8–13.

12. Nelson NC. Rapid detection of genetic mutations using the chemiluminescent hybridization protection assay (HPA): overview and comparison with other methods. *Critical Reviews in Clinical Laboratory Sciences*, 1998, 35(5):369–414.

13. Dhingra K et al. Hybridization protection assay: a rapid, sensitive, and specific method for detection of Philadelphia chromosome-positive leukemias. *Blood*, 1991, 77(2):238–242.

14. Tsongalis GJ. Branched DNA technology in molecular diagnostics. *American Journal of Clinical Pathology,* 2006, 126(3):448–453.

Annex 4

Media, reagents, diagnostic tests, and stains (recipes)

NOTE: The reagents for media and stain preparation are listed in alphabetical order by category. Some recipes appear in the chapters and are not repeated in this annex.

A4.1 Transport, storage, and culture media

Amies transport medium (ingredients refer to the commercially available product; Van Dyck et al., 1999)

Charcoal, pharmaceutical neutral	10 g
Sodium chloride	3.0 g
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Potassium chloride	0.2 g
Sodium thioglycolate	1.0 g
Calcium chloride	0.1 g
Magnesium chloride	0.1 g
Agar	4.0 g
Distilled water	1 l

1.   Suspend all ingredients in 1 litre of distilled water.
2.   Boil to dissolve the agar completely.
3.   Distribute into small, screw-capped tubes or bottles, stirring meanwhile to keep charcoal evenly suspended. Sterilize by autoclaving at 120°C for 15 minutes.
4.   Store the media at 2–8°C for up to 6 months.

Brain-heart infusion (BHI) + 20% glycerol storage medium (commercially available)

BHI	3.7 g
Glycerol	20 ml
Distilled water	80 ml

1.   Mix the ingredients in a 150 ml screw-capped bottle, autoclave, and cool to room temperature.
2.   Using a sterile pipette, aseptically dispense 1.0 ml of the solution into 1.5 ml screw-capped polypropylene cryotubes (it is essential to use proper tubes to avoid breakage).
3.   Store the medium at 4°C until use.

Chocolate agar—see GC medium agar base (GCMB) with haemoglobin (under Thayer–Martin medium).

Columbia blood agar

Columbia blood agar base	39 g
Horse blood (defibrinated)	90 ml
Distilled water	1 l

1. Suspend 39 g Columbia blood agar base in 1 litre of distilled water while mixing. Subsequently steam or boil gently until completely dissolved.
2. Autoclave at 121°C for 15 minutes.
3. Allow the agar medium to cool to 70°C in a water-bath.
4. Aseptically add 90 ml of defibrinated horse blood to the agar. Swirl to mix and leave at 70°C for 30 minutes, continuing to gently mix, on occasion, until the blood becomes chocolate brown in colour.
5. Transfer the flask to a 50°C water-bath to cool before dispensing 20 ml volumes in 9-cm Petri dishes.
6. Allow the plates to solidify at room temperature and then store inverted at 2–8°C in sealed plastic bags for up to 4 weeks.

Columbia agar + 1% haemoglobin + activated charcoal + 5% fetal calf serum + 1% IsoVitaleX enrichment + vancomycin (3 µg/ml)

This is recommended for culture of *Haemophilus ducreyi*.

Columbia agar base	40 g
Bovine haemoglobin	10 g
Activated charcoal	2 g
Fetal calf serum	50 ml
IsoVitaleX enrichment	10 ml
Vancomycin solution	3 mg in 5–10 ml of fluid
Distilled water	1 l

1. Weigh the bovine haemoglobin in a flask (A) and add 400 ml distilled water. Allow this to stand in a fridge at 4°C overnight. The following morning shake the flask and suspend all the powder. Use a pipette or stirring bar to suspend any haemoglobin powder sticking to the base of the flask. Bring to room temperature.
2. In a second flask (B), add the Columbia agar, the activated charcoal, and 600 ml of distilled water.
3. Autoclave flasks A and B separately at 121°C for 15 minutes.
4. Cool the flasks to 56°C in a water-bath.
5. With aseptic technique in a laminar flow hood, slowly pour the sterilized bovine haemoglobin solution (flask A) carefully into flask B containing the Columbia agar base and charcoal, pouring onto the internal side of the flask to avoid bubble creation.
6. Aseptically add 50 ml (5%) sterile fetal calf serum, 10 ml (1%) IsoVitaleX enrichment, and a sterile volume of fluid containing 3 mg vancomycin (filter-sterilized if required).
7. Once everything is added, mix the media with a careful swirling movement. Pour the media aseptically into 20 ml petri dishes and let the media set before placing in plastic bags and storing in the fridge at 4°C until needed. Agar plates should be used within 1–2 weeks of preparation.

Diamond's medium (ingredients refer to the commercially available product; Van Dyck et al., 1999)

Diamond's medium is used to culture *Trichomonas vaginalis.* Another commercial culture system is InPouch TV (Annex 5).

Trypticase peptone	20 g
Yeast extract	10 g
Maltose	5.0 g
L-Cysteine hydrochloride	1.0 g
L-Ascorbic acid	0.2 g
Dipotassium hydrogen phosphate	0.8 g
Potassium dihydrogen phosphate	0.5 g
Agar	0.5 g

1. Suspend and dissolve 38 g of the Diamond's medium powder (all the above ingredients) in 900 ml of distilled water.
2. Autoclave and cool the medium to 50°C.
3. Add 100 ml of sheep or bovine serum and 0.1 g/l of chloramphenicol.
4. Store at 4°C and consume within 3 months.

GC medium agar base (GCMB) supplemented with 1% defined growth supplement (Kellogg's defined supplement or IsoVitaleX/Vitox)

GCMB with supplements is recommended for agar dilution, disk diffusion, and Etest (bioMérieux France) methods for *N. gonorrhoeae* antimicrobial susceptibility testing.

GCMB	36 g
IsoVitaleX/Vitox/Kellogg's defined supplement	10 ml
Distilled water	1 l

1. Dissolve 36 g of GCMB in 1 litre of distilled water and autoclave at 121°C for 15 minutes.
2. Allow the agar media to cool to 50°C in a water-bath, add 10 ml of dissolved IsoVitaleX/Vitox or Kellogg's and mix well.
3. Dispense 20–25 ml per 9 cm Petri dish and 60 ml per 14 cm Petri dish.
4. Allow the plates to solidify in room temperature and store inverted at 2–8°C in sealed plastic bags for up to 4 weeks.
5. IsoVitaleX and Vitox can be purchased commercially while Kellogg's defined supplement is prepared in-house (see overleaf).

Kellogg's defined supplement (*1, 2*)

Glucose	40 g
Glutamine	1.0 g
0.5% Ferric nitrate solution	10 ml
Distilled water	90 ml

1. Mix all ingredients in a 150 ml flask.
2. Sterilize by autoclaving.
3. Cool in a 50°C water-bath.
4. Add 1 ml of sterile 20% cocarboxylase solution (filtered not autoclaved).
5. Aseptically dispense the solution in a 100 ml screw-capped storage bottle and store at 4°C (stable for several months).

GCMB + 2% haemoglobin + 5% fetal calf serum + 1% IsoVitaleX enrichment + vancomycin (3 µg/ml)

This is recommended for culture of *H. ducreyi*.

GCMB	36 g
Bovine haemoglobin	20 g
Fetal calf serum	50 ml
IsoVitaleX enrichment	10 ml
Vancomycin solution	3 mg in 5–10 ml of fluid
Distilled water	1 l

1. In a 2-litre flask (A), add the bovine haemoglobin to 500 ml of distilled water and stir well to mix.
2. In another 2-litre flask (B), add the GCMB to a further 500 ml of distilled water and heat on a stir plate until boiling.
3. Autoclave both flasks at 121°C for 30 minutes then cool to 56°C.
4. With aseptic technique in a laminar flow hood, slowly pour the sterilized bovine haemoglobin solution (flask A) carefully into flask B containing the GCMB, pouring onto the internal side of the flask to avoid bubble creation.
5. Add 50 ml (5%) sterile fetal calf serum, 10 ml (1%) IsoVitaleX enrichment, and a sterile volume of fluid containing 3 mg vancomycin (filter-sterilized if required).
6. Mix the medium with a careful swirling movement.
7. Pour the media aseptically into 20 ml Petri dishes and let the media set before placing in plastic bags and storing in the fridge at 4°C until needed.
8. Agar plates should be used within 1–2 weeks of preparation.

Iscove's modified Dulbecco medium (IMDM-VGA) (commercially available, Sigma-Aldrich, Life technologies, USA)

Kupferberg medium (ingredients refer to the commercially available product; Van Dyck et al., 1999)

Tryptose (or trypticase)	20 g
Maltose	1.0 g
L-Cysteine hydrochloride	1.5 g
Agar	1.0 g
Methylene blue	0.003 g
Chloramphenicol	0.1 g
Yeast extract	0.1 g

1. Suspend 23.7 g of the Kupferberg medium powder in 950 ml of distilled water, autoclave, and cool to 50–55°C.
2. Add 50 ml of serum (bovine, human, sheep, rabbit, or horse), 1 000 000 IU of penicillin, and 150 mg of streptomycin.

Liquid cytology medium (commercially available, BD Diagnostics)

Modified Eagle's medium (MEM-VG) (commercially available, Sigma-Aldrich, Gibco Life Technologies, USA)

Modified Thayer–Martin medium

Prepare GCMB with haemoglobin and supplements (see Thayer–Martin medium). Add selective antimicrobial mixtures, which are commercially available: VCAT (vancomycin, colistin, amphotericin B or anisomycin, trimethoprim lactate), VCNT (vancomycin, colistin, nystatin, trimethoprim lactate) or LCAT (lincomycin, colistin, amphotericin B or anisomycin, trimethoprim lactate). These supplements should be hydrated as recommended by the manufacturer and added to the medium (*3*, *4*). The medium is also commercially available.

Mueller-Hinton broth (commercially available)

Ingredients in commercial preparations:

Beef extract	2.0 g
Acid digest of casein	17.5 g
Soluble starch	1.5 g

1. Suspend 21 g of dehydrated medium in 1 litre of distilled water.
2. Mix and dispense in small aliquots (1.5 or 2 ml) and autoclave.

Mueller–Hinton agar + 5% chocolatized horse blood + 1% IsoVitaleX enrichment + vancomycin (3 µg/ml)

This is recommended for the culture of *H. ducreyi*.

Mueller–Hinton agar base	36 g
Horse blood	50 ml
Fetal calf serum	50 ml
IsoVitaleX enrichment	10 ml
Vancomycin solution	3 mg in 5-10 ml of fluid
Distilled water	1 l

1. Weight out 38 g of Mueller–Hinton agar base and make up to 1 litre with distilled water, autoclave, and cool to 56°C.
2. Add 50 ml (5%) of sterile horse blood when the dissolved agar base reaches 56°C and mix.
3. Chocolatize the suspension by placing the flask in a 70°C water-bath for approximately 15 minutes.
4. Cool to 56°C and then add 50 ml (5%) sterile fetal calf serum, add 10 ml (1%) IsoVitaleX enrichment, and a sterile volume of fluid containing 3 mg vancomycin (filter-sterilized if required).
5. Mix the medium with a careful swirling movement.
6. Pour the media aseptically into 20 ml Petri dishes and let the media set before placing in plastic bags and store in the fridge at 4°C until needed.
7. Agar plates should be used within 1–2 weeks of preparation.

Mycoplasma broth base (*5*) (medium is commercially available)

Beef heart infusion	50 g
Peptone	10 g
NaCl	5.0 g
Distilled water	1 l

1. Mix the ingredients.
2. Autoclave at 121°C for 15 min and store at 4°C.

New York City agar (commercially available; Van Dyck et al., 1999)

Prepare GCMB with supplements and add laked horse blood, yeast autolysate mixture, and VCAT inhibitor.

Yeast autolysate mixture contains:

Yeast autolysate	5.0 g
Glucose	0.5 g
Sodium bicarbonate	0.075 g

Laked horse blood: lyse by freeze and thaw or by adding 5 ml/l of saponin to GCMB medium.

1. Suspend 18 g of GCMB in 430 ml of distilled water, mix, and boil.
2. Autoclave and cool to 50–55°C.
3. Add 50 ml of laked horse blood, yeast autolysate mixture, and VCAT inhibitor (according to manufacturer's instructions).
4. Mix and pour into Petri dishes.

Sabouraud dextrose agar (*5*)

Glucose	40 g
Neopeptone or polypeptone (commercially available)	10 g
Agar	15–20 g
Demineralized water	1 l

1. Heat the mixture to dissolve completely. Final pH 5.6.
2. Dispense into tubes (18–25 mm in diameter) and autoclave at 121°C for 15 minutes.

If chloramphenicol is to be added to the medium, add appropriate volume and concentration.

Skim milk medium (Van Dyck et al., 1999)

Dried milk powder	100 g
Distilled water	1 l

1. Suspend 100 g of dried milk powder in 1 litre of distilled water.
2. Autoclave for 15 minutes at 112–115°C.
3. Store at 4–8°C.

Note: It is important to avoid overheating, otherwise caramelization will occur.

Stuart transport medium (also commercially available)

Sodium thioglycolate	1.0 g
Sodium glycerophosphate	10.0 g
Calcium chloride	0.1 g
Methylene blue	0.002 g
Cysteine hydrochloride	0.5 g
Agar	3–5.0 g
Distilled water	1 l

1. Mix the ingredients in distilled water and bring to boil.
2. Distribute into small screw-capped bottles after adjusting the pH to 7.3–7.4.
3. Autoclave at 121°C for 15 minutes and immediately tighten the cap.
4. The medium should be colourless when cool.
5. Store in a refrigerator at 4–8°C.
6. If the colour changes to blue on storage, the medium is aerated and therefore unfit for use.
7. Loosen the screw-cap and heat the medium to remove the trapped air.

Sucrose phosphate transport medium (2SP) with antibiotics (ingredients refer to the commercially available product; Van Dyck et al., 1999)

Dipotassium hydrogen phosphate	2.1 g
Potassium dihydrogen phosphate	1.1 g
Sucrose	68.5 g
Distilled water	1 l

1. Combine ingredients, bring pH to 7.2 and filter to sterilize.
2. Aseptically add supplements (see below) to 90 ml aliquots.

Supplements:

Fetal calf serum	10 ml
Gentamicin	10 mg
Vancomycin	10 mg
Amphotericin B	0.5 mg

Sucrose-phosphate-glutamate (SPG) storage medium

Sucrose	75 g
KH_2PO_4	0.6 g
K_2HPO_4	2.83 g
Add water to	1 l

1. Adjust pH to 7.4 with 2 N NaOH, if necessary.
2. Aliquot into 100 ml bottles and autoclave for 20 minutes.
3. Store up to 1 year at 4°C.

Thayer–Martin medium

GCMB (ingredients in commercial preparation)—chocolate agar

Peptone	15.0 g
Corn starch	1.0 g
Dipotassium hydrogen phosphate	4.0 g
Potassium dihydrogen phospate	1.0 g
Sodium chloride	5.0 g
Agar	10.0 g
Haemoglobin: a dried powder of bovine haemoglobin	10.0 g
IsoVitaleX, Vitox, or Kellogg's supplement enrichment	10.0 ml
Distilled water	1 l

1. Dissolve 36 g of GCMB (Difco, BD) in 500 ml of distilled water, let it sit until powder dissolves completely. If needed, use a magnetic stirring bar.
2. Suspend 10 g of haemoglobin in 500 ml of distilled water, make a paste before adding the water, mix thoroughly to dissolve, and bring to boil.
3. Sterilize both the solutions by autoclaving at 121°C for 15 minutes. Cool by placing the flasks in 50°C water-bath.
4. Remove each flask from the water-bath and aseptically add the haemoglobin solution to the GCMB flask.

5. Aseptically add 10 ml of dissolved defined supplement (e.g. Kellogg's defined supplement, IsoVitaleX, or Vitox).

6. Mix the contents completely with a gentle swirling motion.

7. Immediately pour 20–25 ml of the mixture into each Petri dish (9 cm).

8. Allow the media to solidify at room temperature.

9. Store plates inverted at 2–8°C in sealed plastic bags for up to 3 weeks.

IsoVitaleX and Vitox can be purchased commercially while Kellogg's defined supplement is prepared in-house (see GCMB).

To the chocolate agar, add vancomycin, colistin, and nystatin inhibitor (VCN).

VCN formula:

Vancomycin	300 µg/ml
Colistin	750 µg/ml
Nystatin	1250 units/ml

Modified Thayer–Martin is made by adding trimethoprim lactate at a concentration of 5.0 µg/ml.

Tryptic soy broth (Van Dyck et al., 1999)

Acid digest of casein (tryptone)	17 g
Soya peptone	3 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Glucose	2.5 g
Distilled water	1 l

1. Suspend 36 g of the commercial powder in 1 litre of distilled water; pH 7.3.

2. Mix well and dispense 2 ml aliquots into small tubes.

Viral transport medium (Van Dyck et al., 1999)

The medium consists of Hank's balanced salt solution (HBSS, commercially available), supplemented with amphotericin B, bovine serum albumin, and gentamicin.

HBSS (commercially available)	10.3 g
Amphotericin B	5.0 mg
Bovine serum albumin 10%	100 ml
Gentamicin	50 mg
Distilled water	1 l

1. Dissolve 10.3 g of HBSS powder in 1 litre of distilled water.

2. Add amphotericin B, bovine serum albumin (dissolved in distilled water), and gentamicin.

3. Mix and adjust the pH to 7.3 with sodium bicarbonate, 7.5% solution.

A4.2 Reagents and diagnostics tests

API NH—*Neisseria gonorrhoeae*

As the API NH procedures require an inoculum equivalent to a 4.0 McFarland standard, it is generally necessary to subculture the isolate prior to testing to obtain sufficient inoculum. The following media may be used to culture *N. gonorrhoeae* before using the API NH strip: chocolate agar with 2% IsoVitaleX or its derivative (i.e. Thayer–Martin) with or without antibiotic; supplemented GCMB may also be used. Blood-based agar media (Columbia blood agar base, trypticase-soy, New York City medium) may be used, although the strength of certain biochemical reactions will be modified (this should be taken into account when reading the reaction; please see the manufacturer's instructions). An alternative to API NH is also the RapID NH (Remel).

1. Record the isolate number on the strip.
2. Place the strip in the incubation box.
3. Using a swab, pick up a few well-isolated colonies and prepare a suspension (in the NaCl provided with the kit) with turbidity equivalent to 4.0 McFarland standard, ensuring it is well mixed using a vortex mixer. Use 18- to 24-hour culture. The suspension must be used immediately after preparation.
4. Distribute the prepared bacterial suspension (approximately 50 µl) in the first 7 microtubes (PEN to URE, inclusive). Avoid the formation of bubbles.
5. Fill the last 3 microtubes with the suspension (approximately 150 µl), avoiding the formation of a convex meniscus.
6. Overlay the first 7 microtubes (PEN to URE, inclusive) with mineral oil (provided with the kit).
7. Incubate for 2 hours at 36°C under aerobic conditions.
8. After the incubation period, read the reactions by referring to the reading table included in the kit (bioMérieux available at www.biomerieux.com).

Reference strains:
See manufacturer's recommendation (bioMérieux, France) for quality control. Use *N. gonorrhoeae* ATCC 31426 or a 2008 WHO *N. gonorrhoeae* reference strain.

Buffers

Type of solution	Concentration (mol/l)	Weight per litre of stock solutions		pH	Volumes used to prepare 100 ml of working solution (ml)	
		A	B		A	B
Phosphate buffer	0.025	3.41 g of KH_2PO_4	4.46 g of $Na_2HPO_4 \times 2H_2O$	6.8	50.8	49.2
	0.1	13.62 g of KH_2PO_4	17.82 g of $Na_2HPO_4 \times 2H_2O$	6.9	43.9	56.1
Sörensen's citrate-sodium hydroxide buffer	0.01	Dissolve 2.1 g of citric acid mono-hydrate in 20 ml of 1 mol/l NaOH and add distilled water to 1 litre (= 0.01 mol/l disodium citrate)	0.01 mol/l HCl	5.6	69.3	30.7

β-lactamase tests

Several tests may be used to determine whether an isolate produces β-lactamase (e.g. iodometric, phenol red) (*3*). The easiest is the chromogenic cephalosporin (nitrocefin) test. See Chapter 4 for details.

Carbohydrate utilization tests

Ingredients for cysteine trypticase agar (CTA) (Difco, BD, USA):

Tryptone	20 g
L-Cysteine	0.5 g
Sodium chloride	5.0 g
Sodium sulfite	0.5 g
Agar	2.5 g
Phenol red	17 mg

1. Suspend 28.5 g of CTA powder in 1 litre of distilled water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at not more than 118°C for 15 minutes.
4. Add 5–10 g of carbohydrate, i.e. glucose, maltose, sucrose, or lactose, before autoclaving or dissolve medium in 900 ml water, autoclave, and aseptically add 100 ml of sterile 5–10% carbohydrate solution.
5. Mix the contents of the flask with a gently swirling motion and distribute into pre-sterilized 10 ml screw-capped tubes aseptically.
6. Cool the tubes in a slanted position to provide a slope and allow the medium to solidify at room temperature.
7. Store tubes at 4°C.
8. Test the prepared medium for performance using stable, typical control cultures as specified by the manufacturer (Difco, BD, USA).

Rapid carbohydrate utilization test (RCUT)

The following reagents are commercially available:

> Lysed blood agar (LBA) medium with 0.5% glucose
> Buffered balanced salt indicator solution (BSS)
> Carbohydrate solutions:
>> 10% glucose (G)
>> 10% lactose (L)
>> 10% sucrose (S)
>> 10% maltose (M)
> Ampicillin solution (200 mg/ml) for β-lactamase production test

A pure culture for the identification of suspected *N. gonorrhoeae* is obtained by subculturing a single colony on an LBA medium containing 0.5% glucose.

1. Emulsify 2 full 10 μl loops of the isolate from a pure overnight growth culture into a tube containing 1.5 ml BSS and mix well with a Pasteur pipette to obtain 10^9 organisms per ml.
2. Mark 6 wells of a microtitre plate as 6 wells as C (control), G, L, M, S, and P'ase (penicillinase).
3. Add 25 mL of 10% sterile carbohydrate solution to the G, L, M, S wells and 25 ml of ampicillin solution to the P'ase well. The first well without any sugar will serve as a control.
4. Add 100 ml (4 drops) of bacterial suspension to each of the six wells.

5. Read after 2–4 hours of incubation at 35–37°C in air (not in CO_2). It is recommended that the β-lactamase reaction is examined again after 24 hours as slow β-lactamase reactions occur with occasional strains.

Interpretation:

As *N. gonorrhoeae* utilizes glucose (not maltose, sucrose and lactose), only the tube containing glucose should have a colour change.

The results of carbohydrate utilization and β-lactamase production are recorded as follows:

Control tube/well   =   Red
Yellow colour   =   Positive reaction
Orange-red colour   =   Negative reaction

Note: If *N. gonorrhoeae* is suspected and a doubtful result is obtained with the rapid carbohydrate utilization test, check the purity of the culture and confirm identity by using other tests such as serological tests.

Glucose-potassium-sodium-phosphate (GKNP) solution

This is HBSS without Ca^{++} and Mg^{++} (commercially available)

10× stock

NaCl	80.0 g
Glucose	10.0 g
KCl	4.0 g
KH_2PO_4	0.60 g
Na_2HPO_4	0.48 g
Phenol red stock (optional)	100 ml
H_2O	900 ml

1. Mix ingredients.
2. Autoclave and store 10× stock at 4°C until use; stock solution expires after 6 months.
3. Working solution is made by diluting the 10× stock 1:10 with H_2O.

Gonochek-II

The Gonochek-II (TCS Biosciences, Ltd.) is a growth independent test used to distinguish Neisseria species by their ability to hydrolyse three enzymes specifically produced by a species: proline iminopeptidase/prolyliminopeptidase (PIP) for *N. gonorrhoeae*, γ-glutamyl-aminopeptidase for *N. meningitidis,* and β-galactosidase for *N. lactamica.*

The Gonochek-II kit comprises a single tube containing three chromogenic substrates. The hydrolysis of these substrates produces a distinct colour. The specific colour produced relates to the enzyme present, and therefore indicates the presence of *N. gonorrhoeae, N. meningitidis,* or *N. lactamica,* respectively.

Other commensal Neisseria species may produce PIP, so enzymatic tests should be performed on strains grown on selective media. PIP-negative *N. gonorrhoeae* strains have also been reported (*6–8*); therefore, specimens should be confirmed using an alternative method (*3*).

GonoGen-II

GonoGen-II (New Horizons Diagnostics Corporation) is a monoclonal antibody-based colorimetric test in which mono-clonal antibodies against the PorB protein (PorB IA and PorB IB) of *N. gonorrhoeae* have been pooled and adsorbed

to suspended metal-sol particles. GonoGen II uses a solubilizing buffer to strip the cell wall from the test organism, thereby exposing the PorB protein. A pool of monoclonal antibodies linked to a red metal-sol carrier is used to detect the antigens specific to *N. gonorrhoeae.* The subsequent antigen-antibody complex is detected by a filtration device giving rise to a clear-cut red dot endpoint (*3*).

1. Label a separate test tube for each isolate and the control strains.
2. Dispense 0.5 ml of solubilizing buffer and adjust McFarland turbidity to 1.0 by collecting colonies from selective or enriched agar.
3. Vigorously shake or vortex the GonoGen-II reagent.
4. Add one drop of GonoGen-II reagent into each of the tubes to be tested. Mix well.
5. Allow tubes to stand for at least 5–15 minutes (longer reaction times increase the clarity of the reaction).

Interpretation:
Positive: indicates *N. gonorrhoeae*, pink to red dot in the well.
Negative: not *N. gonorrhoeae*, white to pale pink dot in the well.

McFarland turbidity standards (*9*)

McFarland turbidity standards are prepared by mixing the following solutions at different ratio to obtain the desired McFarland turbidity scale (for example 0.5, 1, 2 etc.).

McFarland turbidity scale	0.5	1	2	3	4
Anhydrous barium chloride 1% solution (w/v)	0.05 ml	0.1 ml	0.2 ml	0.3 ml	0.4 ml
Sulfuric acid 1% solution (v/v)	9.95 ml	9.9 ml	9.8 ml	9.7 ml	9.6 ml

Tightly seal the tubes and store at room temperature in the dark (these will remain stable for 6 months).
Before use, invert the tubes several times to suspend the barium precipitates.

For comparison, use a background with horizontal black and white stripes.

Oxidase test (for confirmation of identification of *N. gonorrhoeae* after culture):

In-house preparation (Morse et al., 1996 and 2010):

Tetra-methyl-ρ-phenylenediamine dihydrochloride	0.5 g
Distilled water	50 ml

1. Dissolve the substrate in 50 ml distilled water.
2. Place a filter paper in a Petri dish and saturate with the reagent.
3. Pick a portion of the colony to be tested using a platinum wire and rub on the filter paper.

Interpretation:
A positive result is obtained by a deep purple colour appearing within 10 seconds.
Oxidase reagents can be purchased commercially.

Filter paper method:

1. Crush the glass ampoule containing the reagent inside the dispenser by squeezing the sides of the tube.
2. Label filter paper with the number of the test organism and wet the filter paper with the reagent.

3. Pick a colony from an overnight culture with a sterile loop or applicator stick.

4. Rub the inoculum onto the reagent-saturated filter paper.

5. Examine for the appearance of a dark purple colour (positive reaction) within 10–30 seconds.

Direct agar plate method:

1. Add 2–3 drops of oxidase reagent directly onto agar plates containing an overnight culture.

2. Examine for rapid colour change from pink to maroon to dark purple within 10–30 seconds.

Interpretation:

Oxidase positive-organisms produce a purple colour within 30 seconds.

Oxidase negative-organisms produce a light pink colour or remain colourless.

Phadebact monoclonal GC test

In the Phadebact monoclonal GC test, two pools of murine monoclonal antibodies are separately mixed with the Protein A of non-viable *Staphylococcus* which permits the subgrouping of gonococcal isolates into WI (PorB IA) and WII/WIII (PorB IB) groups.

1. Remove fresh grown colonies of *N. gonorrhoeae* that have been presumptively identified and suspend them in 0.5 ml of 0.9% sterile phosphate buffered saline solution (PBS, see below) to a 0.5 McFarland turbidity. Use a test tube with a cap.

2. Place the closed tube in a boiling water-bath for at least 5 minutes and then cool to room temperature.

3. Before using the gonococcal reagent, shake thoroughly. Put one drop of the WI and one drop of the WII/WIII reagents, respectively, on a slide.

4. Add one drop of the boiled gonococcal suspension to the WI reagent and one drop to the WII/WIII reagent. Be sure to include a negative control to which only PBS has been added.

5. Mix the drops thoroughly but gently with a fresh disposable loop. Use a fresh loop for each reagent.

6. Rock the slide and read the result within 1 minute.

Interpretation:

A precipitate with either the WI or WII/WIII gonococcal reagent constitutes a positive result. A positive reaction with both reagents is an equivocal result that will need to be re-tested. If no reaction occurs with either the WI or WII/WIII gonococcal reagent, the test result is negative (a negative result suggests that the isolates tested are not *N. gonorrhoeae*).

Phosphate buffer (see Buffers)

Phosphate buffered saline solution (PBS)

PBS can be prepared in-house (see below) and is also commercially available in pre-prepared powder or tablets.

To prepare 1 litre of 1 × PBS solution mix:

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.78 g
Potassium dihydrogen phosphate	0.27 g
Distilled water	1 l

1. Dissolve the ingredients in 80 ml of distilled water.
2. Adjust pH as required (7.4, 6.8, etc.).
3. Adjust volume to 1 litre with additional distilled water.
4. Sterilize by autoclaving.

Sörensen's citrate-sodium hydroxide buffer (see Buffers)

A4.3 Stains

Giemsa stain (10%) (*10*)—*Klebsiella granulomatis* (Donovan bodies)

Stock solution:

Giemsa powder	0.5 g
Glycerol	33 ml
Methanol absolute, acetone free	33 ml

1. Dissolve the Giemsa powder in the glycerol by placing the mixture in a 55°C water-bath for 90 minutes.
2. When crystals are dissolved, add absolute methanol.
3. Store at room temperature.

Working solution (needs to be prepared at the time of use):

Stock solution	1 ml
Phosphate buffer	23 ml

Phosphate buffer:

Solution 1		Solution 2	
Na_2HPO_4	9.47 g	KH_2PO_4	9.08 g
Make up to 1 litre with distilled water		Make up to 1 litre with distilled water	

1. Mix 72 ml of solution 1 with 28 ml of solution 2.
2. Add 900 ml of distilled water.

Staining procedure:

1. Air-dry the smear, fix with absolute methanol for at least 5 minutes, and let dry again.
2. Cover with the working Giemsa solution for 1 hour.
3. Rinse rapidly with 95% ethyl alcohol to remove excess dye.

Gram stain

The Gram stain procedure is described in Chapter 4 and also is reported in this annex as this stain procedure also is used for other pathogens. The reagents can be purchased commercially or prepared in-house (Van Dyck et al., 1999). Ready-to-use kits are also commercially available.

Crystal violet solution (primary stain)
Iodine solution (mordant stain)
Acetone-ethanol (decolorizing agent)
Safranin solution (counter stain)

1. Cover the fixed smear with crystal violet for 30 seconds. Gently rinse with cold tap water.
2. Flood the slide with iodine solution for 30 seconds. Gently rinse with cold tap water.
3. Decolorize with acetone, acetone-ethanol, or 95% ethanol alone until the purple colour stops flooding out of the smear. It is best to hold the slide, in a gloved hand, near running water. The time of discoloration will depend on which agent is used and the thickness of the smear and will be shortest (typically a few seconds) for acetone and require longer (up to a minute) for ethanol. Excessive discoloration must be avoided as Gram-positive bacteria may appear as Gram-negative. Disregard the thick portions of an uneven smear, which may stain blue.
4. Rinse quickly under running water to stop the discoloration and drain off excess water.
5. Counterstain with safranin or fuchsin for 1 minute.
6. Rinse with running water and gently blot the slide with absorbent paper.

Crystal violet solution:

Solution A (10%)		Solution B (10%)	
Crystal violet powder	2 g	Ammonium oxalate	0.8 g
Ethanol 95%	20 ml	Distilled water	80 ml

Mix solutions A and B to produce crystal violet staining reagent. Store for 24 hours and filter through paper.

Acetone-ethanol (1:1 ratio):

Acetone	50 ml
Ethanol 95%	50 ml

Iodine solution:

Potassium iodide	2 g
Iodine crystals	1 g
Distilled water	300 ml

Most references suggest grinding iodine and potassium iodide in a mortar.

Add water slowly as the chemicals are being ground until the iodine is dissolved.

Safranin solution:

Stock solution		Working solution	
Safranin	5 g	Stock solution	10 ml
Ethanol 95%	100 ml	Distilled water	90 ml

Leishman stain

The Leishman stain can be used as an alternative to Giemsa stain for *Klebsiella granulomatis* (donovanosis, Chapter 13) after fixation of the material on the slide with methanol.

Stock solution:

Leishman powder	1.5 g
Methanol	1 l

1. Add Leishman powder to the methanol and mix well, using a few glass beads.
2. Allow the stock solution to stand at room temperature for 24 hours.

Working solution:

| Stock solution | 1 part |
| Buffered water | 2 parts |

1. Prepare a fresh daily working solution by diluting 1 part of stock solution in 2 parts of distilled water (buffered water with 3.76 g/l of disodium hydrogen phosphate and 2.10 g/l of potassium dihydrogen phosphate, pH 7.0–7.2).
2. Store in a tightly closed bottle to prevent moisture entering the stock solution.

Method:

1. Prepare a smear from the ulcer edges (where the Donovan bodies are most likely to be found) on a glass slide.
2. Cover the slide with the working solution Leishman stain for 10 minutes or up to 30 minutes (Giemsa stain).
3. Rinse the slide in buffered water or phosphate-buffered saline solution (pH 7.0–7.2).
4. Leave the slide air dry and then examine under light microscope using oil immersion (1000× magnification).

Interpretation:

Donovan bodies appear as cocco-bacilli with large vacuoles in the cytoplasm of large histiocytes and occasionally in plasma cells and polymorphonuclear leukocytes. The organisms will appear blue-purple, often surrounded by a clear to acidophilic pink capsule.

Methylene blue stain—*N. gonorrhoeae* (*5*)

Methylene blue	0.3 g
Ethanol	30 ml
Distilled water	100 ml

1. Dissolve dye in ethanol.
2. Add distilled water.
3. Prepare and fix a smear on a glass slide.
4. Flood the slide with the methylene blue stain for 1 minute.
5. Wash the stain off the slide using running tap water.
6. Rinse, air dry, and examine microscopically.

Methylene blue/gentian violet stain—*N. gonorrhoeae* (*11*)

Methylene blue
Gentian violet

The methylene blue (MB)/gentian violet (GV) stain is prepared by adding 4 parts MB with 1 part GV (Huker formula).

1. Prepare a thin smear from a clinical gonococcal swab, and the appropriate control culture, separate labelled glass slides.
2. Heat fix by passing the slide over a flame.
3. Add methylene blue for 30–60 seconds.
4. Rinse, air dry, and examine microscopically.
5. *N. gonorrhoeae* will appear as dark purple cocci or diplococci.

Warthin–Starry silver impregnation stain

1% citric acid:

Citric acid	1 g
Distilled or deionized water	100 ml

Acidulated water:

Distilled or deionized water	500 ml

Add enough of the 1% citric acid to bring the pH to 4.0.

2% silver nitrate solution for developing solution:

Silver nitrate	5 g
Acidulated water	250 ml

1% silver nitrate solution for impregnation:

2% Silver nitrate solution	25 ml
Acidulated water, pH 4.0	25 ml

5% gelatin solution:

Gelatin, high grade	2.5 g
Acidulated water	50 ml

0.15% hydroquinone solution:

Hydroquinone crystals	0.075 g
Acidulated water	50 ml

Developer solution:

2% Silver nitrate solution	12 ml
5% Gelatin solution	30 ml
0.15% Hydroquinone solution	16 ml

A4.4 Acknowledgements

A4.5 References

1. Kellogg DS Jr et al. *Neisseria gonorrhoeae:* virulence genetically linked to clonal variation. *Journal of Bacteriolog*y, 1963, 85:1274–1279.

2. Dillon JA, Tostowaryk W, Pauzé M. Effects of different media and methods of inoculum preparation on results of antimicrobial susceptibility testing of *Neisseria gonorrhoeae* by agar dilution. *Antimicrobial Agents and Chemotherapy*, 1987, 31(11):1744–1749.

3. Dillon JR, Starnino S. *Laboratory manual: identification and antimicrobial susceptibility testing of* Neisseria gonorrhoeae*,* 2nd ed. Co-ordinating Centre for the Gonococcal Antimicrobial Susceptibility Surveillance Program in Latin America and the Caribbean, 2002 (http://www.gasp-lac.net/wp-content/uploads2/ EnglishFull_FINAL-VERSION-1_Mar-27-2012.pdf, accessed 5 April 2013).

4. Young H, Moyes A. An evaluation of pre-poured selective media for the isolation of *Neisseria gonorrhoeae*. *Journal of Medical Microbiology*, 1996, 44(4):253–260.

5. Sarafian SK. Media, reagents, test procedures and stains. In: Morse SA, Moreland AA, Holmes KK, eds. *Atlas of sexually transmitted diseases and AIDS,* 2nd ed. Baltimore, Mosby-Wolfe, 1996:326–332.

6. Brown S et al. Absence of prolyminopeptidase-negative *Neisseria gonorrhoeae* strains in Ontario, Canada. *Canada Communicable Disease Report*, 2008, 34:20–23.

7. Martin IE et al. Identification of prolyliminopeptidase-negative *Neisseria gonorrhoeae* strains in Canada. *Sexually Transmitted Diseases*, 2011, 38(1):40–42.

8. Unemo M et al. Global transmission of prolyliminopeptidase-negative *Neisseria gonorrhoeae* strains: implication for changes in diagnostic strategies. *Sexually Transmitted Infections*, 2007, 83(1):47–51.

9. Benson HJ. *Microbiological applications. A laboratory manual in general microbiology*, 6th ed. Dubuque, Wm C Brown Company, 1994.

10. Pillay A et al. Media, stains, reagents and test procedures. In: Morse SA et al., eds. *Atlas of sexually transmitted diseases and AIDS*, 4th ed. Edinburgh, Saunders/Elsevier, 2010:355–365.

11. Taylor SN, DiCarlo RP, Martin DH. Comparison of methylene blue/gentian violet stain to Gram's stain for the rapid diagnosis of gonococcal urethritis in men. *Sexually Transmitted Diseases*, 2011, 38(11):995–996.

Annex 5

Laboratory supplies

NOTE: Laboratory supplies are listed chronologically as they appear in each chapter. Only the product or reagent manufacturer's name has been indicated. This list is not intended to identify comprehensively every manufacturer. Local distributors are not identified.

Chapter 3. Genital mycoplasmas

- Equipment
    - Centrifuge
    - Electrophoresis device
    - Freezer (–70°C)
    - Fridge (4°C)
    - Polymerase chain reaction (PCR) instrument
    - Real-time PCR instrument.

- Reagents
    - Agarose powder
    - Ethidium bromide
    - PCR reagents
    - Real-time PCR reaction reagents.

- Consumables
    - Filtered pipette tips (1 µl, 10 µl, 100 µl, 1000 µl)
    - Pipettors (10 µl, 200 µl, 1000 µl)
    - Swabs (Dacron or rayon)
    - Test tubes (2 ml, 200 µl)

- Tests (commercially available kits)
    - Nucleic acid amplification tests (NAATs), a transcription-mediated amplification (TMA) research-use-only (RUO) assay (Gen-Probe).

Chapter 4. Gonorrhoea

- Equipment
    - Balance (for weighing)
    - Centrifuge
    - Cryovials
    - Extinction jar (with CO_2-generating envelope or candles)
    - Freezer (–80°C)
    - Fridge (4°C)
    - Gas burner
    - Incubators (at 37°C, 5±1% CO_2)
    - McFarland nephelometric standard (PRO-LAB Diagnostics)

- – Microscope (light microscope and fluorescent microscope)
- – Pipettors (10 µl, 200 µl, 1000 µl)
- – pH meter
- – Plastic ruler (mm) or vernier callipers
- – Steer's replicator (multipoint inoculators or calibrated loop) (CMI-Promex Inc., NJ, USA)
- – Vortex
- – Water-bath.

- Reagents
  - – Antimicrobial discs (Oxoid)
  - – Antimicrobial powders (Sigma-Aldrich or the pharmaceutical manufacturer, if available)
  - – Brain-heart infusion (BHI) broth (Oxoid)
  - – Chocolate agar slopes
  - – Columbia agar base (Oxoid)
  - – Cysteine trypticase agar (CTA) (containing glucose, maltose, and sucrose at a final concentration of 1–2%) (Difco, Becton, Dickinson)
  - – Discs (antibiotics) (Oxoid)
  - – Etest strips (bioMérieux, France)
  - – GC medium base (Difco)
  - – Glycerol (15–20%) (Sigma-Aldrich)
  - – Gram stain (crystal violet, iodine solution, [acetone, acetone–ethanol, or ethanol], safranin) (PRO-LAB Diagnostics)
  - – Isovitalex/Vitox (Oxoid)
  - – Methylene blue (PRO-LAB Diagnostics)
  - – Mueller–Hinton broth (Oxoid)
  - – Nitrocefin (Oxoid)
  - – Oxidase reagent
  - – Selective media (Thayer–Martin, modified Thayer–Martin, and New York City)
  - – These media may be commercially obtained from local suppliers or prepared in-house
  - – Transport medium, non-nutritive (Amies or Stuart) (Sigma-Aldrich).

- Consumables
  - – Cryovials
  - – Glass slides
  - – Graduated pipettes
  - – Microbank fluid with cryobeads
  - – Microtitre plates
  - – Pasteur pipettes
  - – Petri dishes (90 mm)
  - – Pipette tips (1 µl, 10 µl, 200 µl, 1000 µl)
  - – Plastic clear tubes
  - – Plastic loop (1 µl 10 µl)
  - – Polycarbonate screw-cap Bijou bottle
  - – Racks for tubes (15 ml or 50 ml) and for cryovials (2 ml)
  - – Swabs (Dacron or rayon).

- Tests (commercially available kits)
    - Abbott RealTime CT/NG (Abbott Molecular)
    - API NH (bioMérieux)
    - APTIMA Combo 2 (Gen-Probe)
    - Chromogenic cephalosporin test (nitrocefin discs)
    - Cobas Amplicor CT/NG (Roche Diagnostics)
    - Gonochek-II (E-Y Laboratories)
    - GonoGen II (New Horizons Diagnostics Corporation)
    - Hybrid Capture 2 (HC2) CT/NG (Digene Corporation)
    - MicroTrak (Trinity Biotech PLC Co)
    - Mueller–Hinton broth (Oxoid)
    - Oxidase test (BACTIDROP oxidase)
    - PACE 2 (Gen-Probe)
    - Phadebact monoclonal GC test (Boule Diagnostics)
    - ProbeTec ET (Becton, Dickinson)
    - ProbeTec GC Q (Becton, Dickinson)
    - RapID NH (Remel-Thermo Fisher Scientific)
    - Selective medium (Thayer–Martin, modified Thayer–Martin, and New York City) (Oxoid).

Chapter 5. Chlamydial infections

- Equipment
    - Balance (for weighing)
    - Biohazard containment hood
    - Centrifuge
    - Cryovials
    - Fluorescence microscope
    - Freezer (–80°C)
    - Gas burner
    - Hemocytometer
    - Incubators (one at 37°C and one at 35°C)
    - Multi-channel pipettor
    - Pipettors (10 µl, 200 µl, 1000 µl)
    - Sonicator
    - Vacuum flask
    - Vortex
    - Water-bath.

- Reagents
    - Acetone
    - Amphotericin B
    - Fluorescent-labelled monoclonal antibodies against *C. trachomatis*
    - Glucose-potassium-sodium-phosphate (GKNP) solution
    - Iscove's modified Dulbecco medium (IMDM-VGA) (Invitrogen; Sigma-Aldrich)
    - Sucrose-phosphate (SP) transport medium with serum and antibiotics
    - Sucrose-phosphate-glutamate (SPG) storage medium.

- Consumables
  - 200 μl pipette tips
  - Calibrated pipettes
  - Cryovials
  - Glass coverslips
  - Glass slides
  - McCoy cell line (McCoy B; ATCC CRL-1696)
  - Pasteur pipettes
  - Sealing film
  - Tissue culture flasks
  - Tissue culture plates (96 well)
  - Tissue culture vials (5 ml) containing 13 mm glass coverslip.

Chapter 6. Trichomoniasis

- Equipment
  - Fridge (4°C)
  - Incubator (37°C)
  - Light microscope.

- Reagents
  - Diamond's medium
  - Kupferberg medium.

- Consumables
  - Coverslips
  - Dacron or rayon swabs on aluminium shaft
  - Dacron or rayon swabs on plastic shaft
  - Glass slides.

- Tests (commercially available kits)
  - Point-of-care (POC) test: OSOM Trichomonas Rapid Test (Genzyme Diagnostics) (approved only for female vaginal swabs)
  - Culture kit: InPouch TV culture system (BioMed Diagnostics)
  - NAAT: APTIMA TV (Gen-Probe).

Chapter 7. Bacterial vaginosis

- Equipment
  - Light microscope
  - Speculum.

- Reagents
  - 10% potassium hydroxide (KOH)
  - Gram stain (crystal violet, iodine solution, safranin) (PRO-LAB Diagnostics)
  - Saline or phosphate-buffered saline (PBS).

- Consumables
  - Cotton swabs
  - Glass slides
  - pH indicator paper strips (pH range 3.8–6.0).

- Tests (commercially available kits)
  - Affirm VP III (Becton, Dickinson)
  - BV blue (OSOM)
  - FemExam Card Test (New Rapid Diagnostic Kit) (Litmus Concepts).

Chapter 8. Candidiasis

- Equipment
  - Incubator at 36°C
  - Light microscope.

- Reagents
  - 10% KOH solution
  - Bovine (or horse) serum
  - Gram stain (crystal violet, iodine solution, safranin) (PRO-LAB Diagnostics)
  - Saline or PBS solution
  - Selective Sabouraud dextrose agar with/without chloramphenicol
  - Transport medium (Amies).

- Consumables
  - Cotton swabs
  - Coverslips
  - Glass slides
  - pH paper strips
  - Test tubes.

- Tests (commercially available kits)
  - API 20C (bioMérieux)
  - API ID3 2C (bioMérieux)
  - Vitek Yeast Biochemical Card (bioMérieux).

Chapter 9. Herpes simplex virus (HSV) infections

- Equipment
  - Centrifuge
  - Freezer (–20°C and –80°C)
  - Incubator (36°C, 5% CO_2)
  - Microscope (fluorescence and stereoscopic)
  - Vortex.

- Reagents
    - Acetone
    - Fluorescein isothiocyanate (FITC) (molecular probes)
    - N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (HEPES)
    - PBS solution
    - Sucrose.

- Consumables
    - Cotton-tipped, Dacron, or nylon-flocked swabs
    - Culture tubes
    - FITC or Immunoperoxidase-labelled, type-specific monoclonal antibodies (to be used directly on infected cells)
    - Microscope slides
    - Shell vials or multiwell plates
    - Sterile cotton-tipped Dacron, or nylon-flocked swab on a wooden/plastic/aluminium shaft
    - Sterile needles
    - Vaginal speculum
    - Vials for transport medium.

- Tests
    - NAATs (commercially available but not approved by the United States of America Food and Drug Administration)
    - Rapid POC tests for HSV antigen detection are commercially available but not widely evaluated.

Chapter 10. Syphilis

- Equipment
    - ABI 310 Genetic Analyzer (Applied Biosystems)
    - Binocular microscope with dark field condenser
    - Chemiluminescence device
    - Electrophoresis device
    - Enzyme-linked immunosorbent assay (ELISA) reader
    - Fluorescence microscope
    - Freezer (–70°C)
    - Fridge
    - Sonicator
    - Water-bath.

- Reagents
    - Acetone
    - Anti-goat IgG or IgM labelled with alkaline phosphatase
    - Biotinylated goat antihuman IgG-labelled with streptavidin-peroxidase
    - Charcoal
    - Fluorescein
    - Fluorescein-conjugate
    - Fluorescein-labelled anti *Treponema pallidum* globulin
    - Horseradish peroxidase
    - Immersion oil

- – Paraffin
- – PBS solution
- – Recombinant proteins for line immunoassays (TpN47, TpN17, TpN15 and TmpA)
- – Toluene red.

- Consumables
  - – Bacteriological loops or stainless steel spatula
  - – Cotton swabs
  - – Microscope slides
  - – Microtitre plates
  - – Nitrocellulose immunochromatographic strips
  - – Pipettes
  - – Strips of nitrocellulose membrane
  - – Syringes (2 ml or 5 ml)
  - – Tests (commercially available kit and in-house test preparation).

- Tests
  - – Serological tests (non-treponemal or reagin tests)
    - Rapid plasma reagin (RPR) test (Becton, Dickinson)
    - Toluidine red unheated serum test (TRUST; New Horizon Diagnostics)
    - Venereal Disease Research Laboratory (VDRL) test
    - Wassermann reaction (WR) test
  - – Serological tests (treponemal)
    - Fluorescent treponemal antibody absorption (FTA-Abs)
    - *T. pallidum* haemagglutination assay (TPHA)
    - *T. pallidum* passive particle agglutination (TPPA) (Fujirebio)
    - Treponemal enzyme immunoassay (EIA) and chemiluminescence assays (CIAs)
    - Treponemal western blot (WB) assays
  - – Rapid POC syphilis tests
    - These tests are formatted as:
    - 1) Lateral flow strip tests (see Chapter 10 for procedure)
    - 2) Flow-through devices (Span Diagnostics, Ltd.)
  - – Dual, rapid non-treponemal/treponemal tests (Chembio Diagnostic Systems, Inc.).

Chapter 11. Lymphogranuloma venereum (LGV)

- Test
  - – Molecular assays to distinguish non-LGV and LGV strains are based on the detection of *pmpH* gene, which is present only in LGV isolates.

Chapter 12. Chancroid

- Equipment
  - Centrifuge
  - Freezer (–70°C)
  - Incubator (32–34°C, water-saturated atmosphere, or 5% CO_2)
  - Light microscope
  - McFarland nephelometric standard (PRO-LAB Diagnostics)
  - Water-bath.

- Reagents
  - Detection of alkaline phosphatase (n-butanol, Sörensen's citrate-sodium hydroxide buffer [0.01 mol/l], 2,6-dibromoquinone-4-chlorimide in methanol [5 g/l], phenol-free disodium phosphate)
  - Gram stain reagents (see Annex 4)
  - Nitrate reduction test (sodium nitrate solution [0.5 g/l], phosphate buffer, pH 6.8 [0.025 mol/l], acetic acid [5 mol/l], α-naphthylamine [5 g/l])
  - Oxidase test (tetramethyl-p-phenylenediamine hydrochloride)
  - Porphyrin test (magnesium sulfate solution, phosphate buffer, pH 6.9 [0.1 mol/l], δ-aminolevulinic acid hydrochloride [2 mol/l])
  - Growth media (see Annex 4).

- Consumables
  - Bacteriological loops
  - Cotton-tipped swabs
  - Strips of filter-papers.

- Tests (identification of molecular targets)
  - Research-based NAATs.

Chapter 13. Donovanosis (granuloma inguinale)

- Equipment
  - Light microscope
  - Punch biopsy forceps.

- Reagents
  - Ethanol 95%
  - Giemsa staining reagents (see Annex 4)
  - Leishman's stain reagent (see Annex 4)
  - Warthin-Starry silver impregnation stain (see Annex 4)
  - Paraffin.

- Consumables
  - Glass slides.

- Tests (identification of molecular targets)
  - Research-based NAATs, target gene *phoE*.

Chapter 14. Human papillomavirus (HPV) infections

- Equipment
    - Balance (for weighing)
    - Centrifuge
    - Electrophoresis devices
    - Fluorescence microscope
    - Freezer (–70°C)
    - Fridge
    - Incubator (37°C)
    - Microwave oven
    - Pipet-aid
    - Ultraviolet transilluminator
    - Vortex
    - Water-bath.

- Reagents
    - Acid acetic
    - Agarose or acrylamide powder
    - Ethidium bromide
    - HPV-specific DNA probes
    - Immersion oil
    - Mercaptoethanol
    - Serological markers.

- Consumables
    - Glass slides
    - Microplate or small tubes for PCR reaction
    - Pipettes (2 ml, 5 ml and 10 ml)
    - Filtered tips (10 µl, 100 µl, 1000 µl)

- Tests (see also Tables 14.2 and 14.3, Chapter 14)
    - HPV DNA detection methods
        - PCR using Hybrid Capture 2 (HC2), HPV test (Qiagen)
        - Care HPV test
        - COBAS 4800 HPV test (Roche)
        - Linear Array HPV genotyping test (Roche)
        - RUO HPV genotyping LQ test (Qiagen)
        - Multiplex HPV genotyping kit (Multimetric)
    - HPV mRNA assay
        - APTIMA HPV test (Gen-Probe)
        - PreTect HPV proofer (Norchip).

Chapter 15. Human immunodeficiency virus (HIV) infections

- Equipment
    - Centrifuge
    - Electrophoresis device
    - ELISA reader and washer
    - Flow cytometer
    - Fluorescence microscope
    - Freezer (–25°C)
    - Fridge
    - Incubator (37°C)
    - Pipet-aid
    - Water-bath.

- Reagents
    - Acrylamide powder
    - Immersion oil
    - Labelled monoclonal antibodies for flow cytometer (anti-CD4, -CD3, -CD8 and -CD45).

- Consumables
    - Blood stabilizers (Cyto-Chex tubes and TransFix)
    - Blood tubes
    - Calibrated pipettes
    - Colour reagent (colloidal gold)
    - Cotton-tipped swabs
    - Filter papers
    - Fluorescent-tagged secondary antibodies
    - Membrane strips (with separated HIV-specific proteins or recombinant proteins or peptides)
    - Slide (for HIV-infected fixed cells).

- Tests
    - Serological tests
        - EIAs (first-, second-, third-, fourth-generation)
        - Rapid tests.
    - Confirmatory assays (serological)
        - Immunofluorescence assays
        - WB assays
        - Line immunoassays.
    - Molecular tests
        - Detection of HIV RNA, DNA, p24 (Commercial kits, NAATs)
        - POC DNA PCR
        - Genotypic HIV drug resistance (HIVDR) testing (TRUGENE and Viroseq)
        - Phenotypic test (highly expensive, not recommended for routine diagnosis).

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