Canadian Public Health Laboratory Network laboratory guidelines for the use of direct tests to detect syphilis in Canada

Raymond SW Tsang PhD[1*], Muhammad Morshed PhD SCCM[2,3], Max A Chernesky PhD FIDSA FAAM FCCM[4], Gayatri C Jayaraman PhD MPH[5], Kamran Kadkhoda PhD FCCM[6,7]

RSW Tsang, M Morshed, MA Chernesky, GC Jayaraman, K Kadkhoda. Canadian Public Health Laboratory Network laboratory guidelines for the use of direct tests to detect syphilis in Canada. Can J Infect Dis Mid Microbiol 2015;26(Suppl A):13A-17A.

Treponema pallidum subsp. *pallidum* and/or its nucleic acid can be detected by various methods such as microscopy, rabbit infectivity test or polymerase chain reaction (PCR) tests. The rabbit infectivity test for *T. pallidum*, although very sensitive, has been discontinued from most laboratories due to ethical issues related to the need for animal inoculation with live *T. pallidum*, the technically demanding procedure and long turnaround time for results, thus making it impractical for routine diagnostic use. Dark-field and phase-contrast microscopy are still useful at clinic- or hospital-based laboratories for near-bedside detection of *T. pallidum* in genital, skin or mucous lesions although their availability is decreasing. The lack of reliable and specific anti-*T. pallidum* antibodies and its inferior sensitivity to PCR may explain why the direct fluorescent antibody test for *T. pallidum* is not widely available for clinical use. Immunohistochemical staining for *T. pallidum* also depends on the availability of specific antibodies, and the method is only applicable for histopathological examination of biopsy and autopsy specimens necessitating an invasive specimen collection approach. With recent advances in molecular diagnostics, PCR is considered to be the most reliable, versatile and practical for laboratories to implement. In addition to being an objective and sensitive test for direct detection of *Treponema pallidum* subsp. *pallidum* DNA in skin and mucous membrane lesions, the resulting PCR amplicons from selected gene targets can be further characterized for antimicrobial (macrolide) susceptibility testing, strain typing and identification of *T. pallidum* subspecies.

Key Words: *Treponema pallidum; Direct detection; PCR; Syphilis*

Les directives du Réseau des laboratoires de santé publique du Canada sur l'utilisation des tests directs pour déceler la syphilis au Canada

Diverses méthodes, telles que la microscopie, le test d'infectivité du lapin et la réaction en chaîne de la polymérase (PCR), permettent de déceler le *Treponema pallidum* sous-espèce *pallidum* et/ou son acide nucléique. Même s'il est très sensible, le test d'infectivité du lapin n'est plus utilisé dans la plupart des laboratoires pour déceler le *T. pallidum*. En effet, des raisons éthiques liées à la nécessité d'inoculer le *T. pallidum* vivant à l'animal, l'intervention exigeante sur le plan technique et la longue attente avant d'obtenir les résultats le rendent peu pratique pour un usage diagnostique régulier. Dans les laboratoires des cliniques ou des hôpitaux, la microscopie à fond noir et la microscopie à contraste de phase contribuent toujours à déceler le *T. pallidum* dans les lésions génitales, cutanées ou muqueuses près du chevet du patient, mais elles sont de moins en moins offertes. Le test d'immunofluorescence directe est peu utilisé pour diagnostiquer le *T. pallidum* en milieu clinique, peut-être en raison de l'absence d'anticorps anti-*T. pallidum* fiables et spécifiques et de sa faible sensibilité par rapport au PCR. La coloration immunohistochimique du *T. pallidum* dépend également de la présence d'anticorps spécifiques, et la méthode est applicable seulement à l'examen histopathologique des prélèvements invasifs de biopsies et d'autopsies. Étant donné les progrès récents des diagnostics moléculaires, la PCR est considérée comme le test le plus fiable, le plus polyvalent et le plus pratique à utiliser en laboratoire. Le PCR est objectif et spécifique pour la détection directe de l'ADN du *Treponema pallidum* sous-espèce *pallidum* dans les lésions de la peau et des muqueuses ; ses amplicons provenant de cibles géniques précises peuvent être caractérisés en vue de tests de susceptibilité antimicrobienne (aux macrolides), du typage des souches et du dépistage des sous-espèces de *T. pallidum*.

INTRODUCTION

Direct testing for the syphilis agent in clinical specimens can be defined as the detection of *T. pallidum* subspecies *pallidum* (subsequently simply refer to as *T. pallidum*) whole bacteria or components in human tissues or fluids. While the diagnosis of syphilis relies primarily on clinical and serological findings, direct testing offers an important diagnostic tool that is particularly useful for the diagnosis of primary and/or congenital syphilis. Because serology can be non-reactive in as many as 30% of cases, especially when testing is performed on subjects with low $CD4^+$ lymphocytes, eg, due to human immunodeficiency virus (HIV) infection (1,2), direct testing in primary syphilis enables earlier diagnosis of disease and prevention of ongoing transmission. In addition to being used for the diagnosis of syphilis, direct amplification of *T. pallidum* genes with subsequent characterization of their nucleotide sequences can serve as an important tool for the detection of antibiotic resistance (3,4), molecular typing of strains (5,6), and identification of nonvenereal *T. pallidum* subspecies *endemicum* and subspecies *pertenue* infections (7,8).

The present review describes the various direct detection methods, compares their usefulness and limitations, and suggests guidelines for when and how these tests may be used for the laboratory investigation of syphilis infection. Several methods are available for the direct detection of *T. pallidum*; these include various forms of microscopy

[1]*National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba;* [2]*BC Public Health Microbiology and Reference Laboratory;* [3]*Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia;* [4]*McMaster University, Hamilton;* [5]*Centre for Communicable Diseases and Infection Control, Public Health Agency of Canada, Ottawa, Ontario;* [6]*Cadham Provincial Laboratory;* [7]*Department of Medical Microbiology & Infectious Diseases and Department of Immunology, University of Manitoba, Winnipeg, Manitoba*

**Denotes section lead*

Correspondence: Dr Raymond SW Tsang, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington Street, Winnipeg, Manitoba R3E 3R2. Telephone 204-789-6020, fax 204-789-2018, e-mail raymond.tsang@phac-aspc.gc.ca

TABLE 1
Comparison of different methods for the direct detection of syphilis, focusing on primary syphilis

Direct test	Historical sensitivity	Historical specificity	Notes
Rabbit infectivity test	Gold standard	Gold standard	Impractical for routine use
Darkfield microscopy	86–97% (13,14)	100% (13,14)	Requires equipment and darkfield microscopy expertise at or in close proximity to the clinical setting
Silver staining	59–60% (49,50)	Prone to false-positive results due to tissue debris	Only for histological tissue section specimens
Direct fluorescent antibody test	Monoclonal antibodies: on lesions: 73–100% (16,17); on tissue: 86–88% (21,22); Polyclonal antibodies: 86–90% (20)	100% (16,17); 97–100% (21,22); (96–97% (20)	No Health Canada approved test available
Immunohistochemistry	74–94% (51,52)	False positive possible with other spirochetal and *Borrelia* infections especially when polyclonal antisera are used	No commercial antibody available
Polymerase chain reaction	Primary syphilis: genital or anal chancres: 78.4%; congenital syphilis, blood: 83% (44)	Approximately 95% except for blood specimens, with lower specificity (44)	No commercial kit available in Canada

using dark field, phase contrast, direct immunofluorescent antibody testing (DIF), immunohistochemical and silver impregnation staining of tissue samples, and polymerase chain reaction (PCR). The rabbit infectivity test was historically considered to be a reference standard, but is rarely available for comparison today. To determine the sensitivity, specificity and predictive values of new tests, a gold standard of more than one existing test is recommended. The different methods are introduced below, along with relative sensitivity, specificity, availability and practicality for clinical use (Table 1).

RABBIT INFECTIVITY TEST (RIT)

This method is neither direct nor rapid, and is technically demanding, with requirements for animal health technicians to maintain and inoculate rabbits with specimens containing live *T. pallidum*. Results can take up to three months to obtain. The test involves injecting clinical specimens intratesticularly into an adult male New Zealand White rabbit shown to be sero-negative for syphilis, and subsequently observing for clinical signs of orchitis and syphilis sero-conversion. Rabbits showing positive clinical signs or serological test results will undergo confirmation testing by dark-field or phase-contrast microscopic examination of an aspirate obtained from their testis.

The RIT has excellent sensitivity and is able to detect as few as one to two treponemal organisms (9). However, ethical concerns regarding inoculating laboratory animals with live pathogenic organisms, the laborious and time-consuming nature of the test, as well as significant costs have rendered the test impractical for routine use. Currently, the test has been discontinued in both federal and provincial public health laboratories. Readers with interest in this test can consult the protocol described by Lukehart and Marra (10).

MICROSCOPIC EXAMINATION

Dark-field or phase-contrast microscopy

Microscopy to detect *T. pallidum* is challenging as the organism is smaller (in width) than most other bacteria, being only 0.1 µm to 0.2 µm in width, and, therefore, cannot be visualized using conventional Gram stain and light microscopy. However, they can be visualized by either dark-field or phase-contrast microscopy, or by special staining such as the silver impregnation stain.

The ideal specimen is serous fluid from genital, skin or mucous membrane lesions with few or no blood cells, which could obscure the detection of spirochetes. However, this method is not suitable for oral and anal lesions because non-pathogenic treponemal organisms from these sites cannot be distinguished from *T. pallidum*. Other specimens that can be examined by dark-field/phase-contrast microscopy for *T. pallidum* include amniotic fluid and lymph node aspirate (11,12).

In order to obtain a specimen with high yield of motile *T. pallidum*, the scab or crust over any lesion should first be removed, and pressure applied at the base of the lesion(s) to express fluid. The fluid is applied onto a clean microscopic glass slide by either touching the glass slide to the moist lesion or by using a loop to transfer the fluid from the lesion to the glass slide. A glass cover slip is placed carefully over the fluid to avoid any air bubbles between the cover slip and the glass slide.

The slide should be examined immediately, either before the slide becomes dry or within 30 min of specimen collection, to maintain the motility of any live *T. pallidum* organisms. The entire slide should be examined methodically using high-dry objective (400× magnification) to look for any spiral organisms with characteristic motility and any suspicious spirochetes observed with an oil immersion objective lens at 1000× magnification. Typical morphology of a motile *T. pallidum* is a slender (0.1 µm to 0.2 µm) corkscrew-shaped spiral organism with an average length of 10 µm and a rotational movement. If the primary lesion is partially healed, dark field microscopy may also be used to examine an aspirate sample from a regional lymph node. Cerebrospinal fluid (CSF) or amniotic fluid can be centrifuged to concentrate the treponemes before dark field/phase contrast microscopic examination. After examination, the specimen or glass slide should be discarded in a container with an appropriate disinfectant, such as 70% alcohol or 10% bleach solution.

The sensitivity of dark-field microscopy for the diagnosis of syphilis is approximately 86% to 97% (13,14) and specificity is 100%. Limitations include: (a) the test cannot be used on oral or anal lesions due to the presence of non-pathogenic spirochetes in these body sites; (b) false-negative results can occur if the patient has received treatment with antibiotics; (c) blood cells and tissue debris can obscure the detection of motile treponemes; (d) the method is subjective and depends on experience of the microbiologist; and (e) sites for specimen collection and testing have to be in close proximity in order for microscopic examination to take place immediately, before the spirochetes loss their motility (15).

Therefore, direct microscopy is most useful for confirmation of a suspected syphilis diagnosis when lesions are visible on skin and mucous membranes (excluding oral and anal sites) for the diagnosis of primary, secondary and early congenital syphilis.

Silver or immunohistochemical staining

Silver impregnation or deposition around *T. pallidum* allows the slender spirochete to be visible in formalin-fixed tissue sections under the light microscope. However, the drawbacks of this method include limited sensitivity, potential false-positive results due to staining artifacts and other normal flora treponemes.

To increase the sensitivity and specificity of direct visualization of *T. pallidum* in clinical specimens, anti-*T. pallidum* antibody is used in either direct fluorescent antibody test (DFA) using fluorescein isothiocyanate (FITC) conjugated antibody (16,17) or in immunohistochemistry (IHC) via avidin-biotin immunoperoxidase reaction (18).

Histological examination of biopsy and autopsy tissues showing positive cellular changes suspected of syphilis should be stained by specific anti-*T. pallidum* antibody in either IHC or DFA test (DFA-TP).

DFA-TP

Direct fluorescent antibodies for *T. pallidum* staining is an alternative to dark-field microscopy. It has the advantage of not requiring live motile organisms to be present in the specimen, and does not require immediate examination after specimen collection. The antibody used may be monoclonal (16) or a polyclonal rabbit antibody to *T. pallidum* absorbed with the Reiter's treponemes to remove cross-reactive antibody to non-pathogenic *Treponema* species. Detection is based on recognition of *T. pallidum* by a specific antibody that is conjugated to FITC in order to provide a fluorescent signal to increase detection sensitivity. The specificity of DFA-TP over dark-field microscopy is accomplished by the use of specific antibody and, therefore, it can be used on specimens taken from oral and anal lesions.

Specimens that can be examined by DFA-TP include: serous fluids from lesions that can be obtained for dark-field or phase-contrast microscopy; other body fluids, such as CSF or aqueous humor fluids, which can be centrifuged at 1500 × *g* for 30 min to concentrate the spirochetes to the sediment, and smears made from the sediment; and biopsy and autopsy formalin-fixed and paraffin-embedded tissue sections. Slides with ulcerative fluids from skin or mucous membrane lesions can be air dried and stored or shipped without fixation at either ambient temperature or at 4°C to 8°C. For long-term storage, slides can be kept and shipped frozen. Body fluids can be stored and shipped at 4°C within a few days of specimen collection. For long-term storage, fluids can be kept and shipped at −20°C.

Positive and negative antigen controls (smears containing dried *T. pallidum* as positive antigen and non-pathogen *Treponema*, such as the Reiter's treponeme, as negative antigen) should be included in every test. Stained smears should be examined first with a 40× objective (400× magnification) and any suspicious fluorescent objects should be verified by the oil immersion 100× objective (1000× magnification) as in the dark-field microscopy for a 2+ or greater fluorescence and a typical morphology for *T. pallidum*.

The DFA-TP test can be applied to any tissue specimens obtained by biopsy or autopsy, including paraffin-embedded tissue sections used for histopathological examination (19). Slides containing paraffin-embedded tissue, have to be deparaffinised, rehydrated, and treated with either phosphate buffered saline containing 1% ammonium hydroxide or with PBS containing 0.25% trypsin. When performing the DFA-TP test on tissue specimens, controls should include: (a) paraffin-embedded tissue sections prepared from *T. pallidum*-infected rabbit testis and processed together with the specimens being tested; and (b) a negative control of non-pathogenic treponemes as a negative antigen control.

The reported sensitivity of the DFA-TP test using monoclonal antibody conjugates on exudates from ulcerative lesions was in the range of 73% to 100%, with specificity of 100% (16,17). The sensitivity and specificity of the DFA-TP test using polyclonal antibody were reported to be 86% to 90% and 96% to 97%, respectively (20). Sensitivity of using DFA-TP on tissue sections is in the range of 86% to 88% and specificity 97% to 100% based on the DFA-TP test using anti-*T. pallidum* monoclonal antibody (21,22). The major limitation for this test is the availability of reliable specific anti-*T. pallidum* antibodies, and potential cross-reactions with other spirochete organisms, especially if polyclonal anti-*T. pallidum* antisera are used.

DETECTION OF *T. PALLIDUM* DNA

In the past two decades, a large number of PCR methods have been described for the detection of *T. pallidum* DNA in a variety of clinical specimens. Reliable and consistent results have been obtained from studies using PCR to detect *T. pallidum* DNA in skin or mucous membrane lesions of syphilis patients (23-28), while PCR on buffy coat, plasma, serum or whole blood is less sensitive (4,26). Other suitable specimens for PCR detection of *T. pallidum* DNA include CSF when neurosyphilis is suspected (29), amniotic fluid for congenital syphilis, placenta or nasal discharges (snuffles) in neonates when early congenital syphilis is suspected (30), and biopsy or autopsy tissue specimens. Castro et al (31,32) have also suggested that scrapings from ear lobes of syphilis patients may provide a positive source of *T. pallidum* DNA detectable by PCR methodologies, with higher detection rates compared with other specimen types.

A number of *T. pallidum* genes have been detected by PCR in patients with syphilis; these include *pol*A, *tpp47*, *bmp* and *tmp*A (33-36). One study comparing *pol*A, *bmp* and *tpp47* as targets for PCR detection appears to suggest that sensitivity of the assay is independent of these targets but dependent on the specimen type (4). In addition, the *arp*, *tpr*, *rps*A (TP0279) and TP0548 genes (5,37,38) have also been detected by PCR in patients with syphilis. Because these latter gene targets are either highly polymorphic or not species or subspecies specific, they are not recommended in general as targets for diagnostic purposes. However, characterization of these PCR amplicons by a combination of restriction enzyme digestion and/or nucleotide sequencing has allowed molecular typing schemes for *T. pallidum* to be established, which can be useful in molecular epidemiological surveys (5,6,37,38). Another application of the PCR amplification of *T. pallidum* genes is in the area of laboratory differential diagnosis of venereal from non-venereal treponemal diseases (7,8). Amplification and characterization of the *T. pallidum* 23S ribosomal RNA gene can identify macrolide resistance in syphilis strains. Resistance to macrolide antibiotics (such as azithromycin and/or spiramycin) in *T. pallidum* has been associated with point mutations in the 23S ribosomal RNA, involving A2058G (39) or A2059G (40) substitutions. With potential increase in macrolide resistance in *T. pallidum* or the lack of information on local prevalence as well as the geographical distribution of resistant strains, a call for an international collaborative effort to enhance global surveillance has recently been made (41).

Specimen types, methods of DNA extraction, PCR platforms and their protocols for amplification of the different target genes for the diagnosis, molecular typing and azithromycin resistance detection in *T. pallidum* have been reviewed recently in a book chapter by Bruisten (42). A protocol for PCR detection of *T. pallidum* DNA in paraffin-embedded specimens is also available (43). A comparison of the published data from January 1990 to January 2012 on the applications of PCR for the study of syphilis infection was recently systematically reviewed (44). This review of the literature confirmed that PCR is most useful for the diagnosis of early syphilis by detection of *T. pallidum*-specific DNA in genital ulcers and skin lesions. Venous blood does not appear to be a suitable specimen type for the PCR diagnosis of syphilis (4,26,45), except in the case of congenital syphilis in neonates (44). In addition, PCR is not as useful as serology in the diagnosis of secondary syphilis (4,27,28).

Although multiplex PCRs (46,47) have been described in the literature for the simultaneous detection and differentiation of genital ulcer diseases caused by herpes simplex virus types 1 and 2, *Haemophilus ducreyi* and *T. pallidum*, their use should be carefully controlled to ensure that sensitivities achieved by the individual assays are not compromised in exchange for convenience and/or cost reduction. The current application of PCRs and other molecular analytical methods for the laboratory investigation of syphilis infections have the most significant impact on: (a) diagnosing early primary syphilis on skin or mucous membrane specimens before seroconversion occurs; (b) confirmation of early congenital syphilis with tests performed on placenta, and non-invasive (discharges) or minimally invasive specimens (blood or skin scrapings) on the newborn infants or neonates born to mothers with positive syphilis serology during gestation; (c) confirmation of neuro-syphilis by testing CSF specimens; (d) detection and surveillance of *T. pallidum*'s resistance to macrolide antibiotics in a population; (e) molecular typing of strains to understand the molecular epidemiology of syphilis infection; and (f) potential differential diagnosis of diseases caused by the different subspecies of *T. pallidum*.

With continuing efforts on comparative genome sequencing of different subspecies of *T. pallidum*, new gene targets will likely be identified that may aid in the molecular identification, typing and diagnosis of diseases caused by the different *T. pallidum* subspecies or other spirochete organisms (48).

SUMMARY

Primary considerations in the assessment of direct testing methods include the test method's performance characteristics (such as test sensitivity and specificity), availability of the test and practicality for clinical use. A comparison of the different detection methods for *T. pallidum* is given in Table 1. As summarized in Table 1, most of the methods involved are either impractical (eg, RIT), not commercially available nor Health Canada licensed (DFA-TP, PCR), or requires special microscope and technical experience in microscopy (dark field or phase contrast microscopy). Therefore, implementation of direct testing for *T. pallidum* in the clinical laboratory would require careful planning and validation of the method and reagents employed. Proficiency testing should also be part of the program to ensure standards and consistency of results. Currently, there are no Health Canada-approved PCR products for the detection of syphilis and laboratories developed their own in-house tests using commercially available reagents and based on peer-reviewed literature. There is also no external proficiency panel available through College of American Pathologists (CAP).

For wider application of PCR assays to diagnose and characterize syphilis infections, studies are needed to improve the diagnostic sensitivity of PCR assays performed on non-ulcerative specimens. To meet this goal, either more sensitive assays, better methods to concentrate pathogens or DNA in clinical specimens are required or better knowledge of where the treponemes may be during the various stages of disease. As more laboratories are using PCR methods for diagnosis of diseases, these issues, ranging from specimen sources, extraction and concentrations of pathogen DNA, PCR platforms, availability of standardised and commercially available kits, and proficiency testing, will become more pressing and may require a coordinated approach to provide solutions.

DISCLOSURES: The authors have no conflicts of interest to declare.

REFERENCES

1. Sparling PF. Natural history of syphilis. In: Holmes KK, Mardh PA, Sparling PF, et al, eds. Sexually Transmitted Diseases, 2nd edn. New York: McGraw-Hill Information Services Co, 1990:213-9.
2. Hicks CB, Benson PM, Lupton GP, Tramont EC. Sero-negative secondary syphilis in a patient with the human immunodeficiency virus (HIV) with Kaposi sarcoma. Ann Intern Med 1987;107:492-5.
3. Stamm LV, Gergen HL. A point mutation associated with bacterial macrolide resistance is present in both 23S rRNA genes of an erythromycin-resistant *Treponema pallidum* clinical isolate. Antimicrob Agents Chemother 2000;44:806-7.
4. Martin IE, Tsang RSW, Sutherland K, et al. Molecular characterization of syphilis in patients in Canada: Azithromycin resistance and detection of *Treponema pallidum* DNA in whole-blood samples versus ulcerative swabs. J Clin Microbiol 2009;47:1668-73.
5. Pillay A, Liu H, Chen CY, et al. Molecular subtyping of *Treponema pallidum* subspecies *pallidum*. Sex Transm Dis 1998;25:408-14.
6. Sutton MY, Liu H, Steiner B, et al. Molecular subtyping of *Treponema pallidum* in an Arizona County with increasing syphilis morbidity: Use of specimens from ulcers and blood. J Infect Dis 2001;183:1601-6.
7. Centurion-Lara A, Molini BJ, Godornes C, et al. Molecular differentiation of *Treponema pallidum* subspecies. J Clin Microbiol 2006;44:3377-80.
8. Fanella S, Kadkhoda K, Shuel M, Tsang R. Local transmission of imported endemic syphilis, Canada, 2011. Emerg Infect Dis 2012;18:1002-4.
9. Magnuson H, Eagle H, Fleischman R. The minimal infectious inoculum of *Spirochaeta pallida* (Nichols strain) and a consideration of its rate of multiplication in vivo. Am J Syph 1948;32:1-18.
10. Lukehart SA, Marra CM. Isolation and laboratory maintenance of *Treponema pallidum*. Curr Protoc Microbiol 2007;12:12A.1.
11. Larsen SA, Johnson RE. Diagnostic tests. In Larsen SA, Pope V, Johnson RE, Kennedy EJ Jr, eds. A Manual of Tests for Syphilis, 9th edn. American Public Health Association, Washington DC, 1998:2-52.
12. Kennedy EJ Jr, Creighton ET. Darkfield microscopy for the detection and identification of *Treponema pallidum*. In Larsen SA, Pope V, Johnson RE, Kennedy EJ Jr, eds. A Manual of Tests for Syphilis, 9th edn. Washington, DC: American Public Health Association, 1998:120-34.
13. Wheeler HL, Agarwal S, Goh BT. Dark ground microscopy and treponemal serological tests in the diagnosis of early syphilis. Sex Transm Infect 2004;80:411-4.
14. Cummings MC, Lukehart SA, Marra C, et al. Comparison of methods for the detection of *Treponema pallidum* in lesions of early syphilis. Sex Transm Dis 1996;23:366-9.
15. Radolf JD, Pillay A, Cox DL. *Treponema* and *Brachyspira*, human host-associated spirochetes. In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW, eds. Manual of Clincal Microbiology, 10th edn, vol 1. Washington, DC: ASM Press, 2011:941-63.
16. Romanowski B, Forsey E, Prasad E, Lukehart S, Tam M, Hook EW. Detection of *Treponema pallidum* by a fluorescent monoclonal antibody test. Sex Transm Dis 1987;22:156-9.
17. Hook III EW, Roddy RE, Lukehart SA, Hom J, Holmes KK, Tam MR. Detection of *Treponema pallidum* in lesion exudates with a pathogen-specific monoclonal antibody. J Clin Microbiol 1985;22:241-244.
18. Phelps RG, Knispel J, Schuman Tu E, Cernainu G, Saruk M. Immunoperoxidase technique for detecting spirochetes in tissue sections: Comparison with other methods. Int J Dermat 2000;39:609-13.
19. Hunter EF, Greer PW, Swisher BL, et al. Immunofluorescent staining of *Treponema* in tissues fixed with formalin. Arch Pathol Lab Med 1984;108:878-80.
20. George RW, Hunter EF, Fears MB. Direct fluorescent antibody test for *Treponama pallidum* (DFE-TP). In: Larsen SA, Pope V, Johnson RE, Kennedy EJ Jr, eds. A Manual of Tests for Syphilis, 9th edn. Washington DC: American Public Health Association 1998:136-46.
21. Schwartz DA, Larsen SA, Beck-Sague C, Fears M, Rice RJ. Pathology of the umbilical cord in congenital syphilis: Analysis of 25 specimens using histochemistry and immunofluorescent antibody to *Treponema pallidum*. Human Pathol 1995;26:784-91.
22. George RW, Hunter EF, Fears MB. Direct fluorescent antibody tissue test for *Treponama pallidum* (DFAT-TP). In Larsen SA, Pope V, Johnson RE, Kennedy EJ Jr, eds. A Manual of Tests for Syphilis, 9th edn. Washington DC: American Public Health Association, 1998:148-56.
23. Wicher K, Noordhoek GT, Abbruscato F, and Wicher V. Detection of *Treponema pallidum* in early syphilis by DNA amplification. J Clin Microbiol 1992;30:497-500.
24. Palmer HM, Higgins SP, Herring AJ, Kingston MA. Use of PCR in the diagnosis of early syphilis in the United Kindgom. Sex Transm Infect 2003;79:479-83.
25. Buffet M, Grange PA, Gerhardt P, et al. Diagnosing *Treponema pallidum* in secondary syphilis by PCR and immunochemistry. J Invest Dermat 2007;127:2345-50.
26. Grange PA, Gressier L, Dion PL, et al. Evaluation of a PCR test for detection of *Treponema pallidum* in swabs and blood. J Clin Microbiol 2012;50:546-52.
27. Heymans R, van der Helm JJ, de Vries HJC, Fennema HSA, Coutinbo RA, Bruisten SM. Clinical value of *Treponema pallidum* real-time PCR for diagnosis of syphilis. J Clin Microbiol 2010;48:497-502.
28. Shields M, Guy RJ, Jeoffreys NJ, Finlayson RJ, Donovan B. A longitudinal evaluation of *Treponema pallidum* PCR testing in early syphilis. BMC Infect Dis 2012;12:353.
29. Hay PE, Clarke JR, Strugnell RA, Taylor-Robinson D, Goldmeier D. Use of the polymerase chain reaction to detect DNA sequences specific to pathogenic treponemes in cerebrospinal fluid. FEMS Microbiol Lett 1990;68:233-8.
30. Genest DR, Choi-Hong SR, Tate JE, Qureshi F, Jacques SM, Crum C. Diagnosis of congenital syphilis from placental examination: Comparison of histopathology, Steiner stain, and polymerase chain reaction for *Treponema pallidum* DNA. Human Pathol 1996;27:366-72.
31. Castro R, Prieto E, Aquas MJ, et al. Detection of *Treponema pallidum* sp. *pallidum* DNA in latent syphilis. Int J STD AIDS 2007;18:842-5.

32. Castro R, Prieto E, Aquas MJ, Manata MJ, Botas J, Pereira FM. Molecular subtyping of *Treponema pallidum* subsp. *pallidum* in Lisbon, Portugal. J Clin Microbiol 2009;47:2510-2.
33. Liu H, Rodes B, Chen CY, Steiner B. 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. J Clin Microbiol 2001;39:1941-6.
34. Kouznetsov AV, Weisenseel P, Trommler P, Multhaup S, Prinz JC. Detction of the 47 kilodalton membrane immunogen gene of *Treponema pallidum* in various tissue sources of patients with syphilis. Diagn Microbiol Infect Dis 2005;51:143-5.
35. Noordhoek GT, Wolters EC, de Jonge ME, van Embden JD. Detection by polymerase chain reaction of *Treponema pallidum* DNA in cerebrospinal fluid from neurosyphilis patients before and after antibiotic treatment. J Clin Microbiol 1991;29:1976-84.
36. Pietravalle M, Pimpinelli F, Maini A, et al. Diagnostic relevance of polymerase chain reaction technology for *Treponema pallidum* in subjects with syphilis in different phases of infection. Microbiologica 1999;22:99-104.
37. Katz KA, Pillay A, Ahrens K, et al. Molecular epidemiology of syphilis – San Francisco, 2004-2007. Sex Transm Dis 2010;37:660-3.
38. Marra CM, Sahi SK, Tantalo LC, et al. Enhanced molecular typing of *Treponema pallidum*: Geographical distribution of strain types and association with neurosyphilis. J Infect Dis 2010;202:1380-8.
39. Lukehart SA, Godomes C, Molini BJ, et al. Macrolide resistance in *Treponema pallidum* in the United States and Ireland. N Engl J Med 2004;351:154-8.
40. Matejkova P, Flasarova M, Zakoucha H, et al. Macrolide treatment failure in a case of secondary syphilis: A novel A2059G mutation in the 23S rRNA gene of *Treponema pallidum* subsp. *pallidum*. J Med Microbiol 2009;58:832-6.
41. Lewis DA, Lukehart SA. Antimicrobial resistance in *Neisseria gonorrhoeae* and *Treponema pallidum*: Evolution, therapeutic challenges and need to strengthen global surveillance. Sex Transm Infect 2011;87:ii39-ii43.
42. Bruisten SM. Protocols for detection and typing of Treponema pallidum using PCR methods. In: MacKenzie CR, Henrich B, eds. Diagnosis of Sexually Transmitted Diseases, Methods and Protocols. Methods in Molecular Biology, 2012;903:141-57. New York: Springer Science + Business Media.
43. Chen CY, Pillay A. Protocol for the detection of *Treponema pallidum* in paraffin-embedded specimens. In: MacKenzie CR, Henrich B, eds. Diagnosis of sexually transmitted diseases, methods and protocols. Methods in Molecular Biology, 2012;903:295-306. New York: Springer Science + Business Media.
44. Gayet-Ageron A, Lauterschlager S, Ninet B, Perneger TV, Combescure C. Sensitivity, specificity, and likelihood ratios of PCR in the diagnosis of syphilis: A systematic review and meta-analysis. Sex Transm Infect 2013;89:251-6.
45. Wang LN, Li J, Huang W, et al. Detection of the *Treponema pallidum* gene and evaluation of treponemal DNA load before and after therapy. Int J STD AIDS 2012;23:e6-e8.
46. Orle AA, Gates CA, Martin DH, Body BA, Weiss JB. Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum* and Herpes simplex virus types 1 and 2 from genital ulcers. J Clin Microbiol 1996;34:49-54.
47. Bruisten SM, Cairo I, Fennema H et al. Diagnosing genital ulcer disease in a clinic for sexually transmitted disease in Amsterdam, The Netherlands. J Clin Microbiol 2001;39:601-5.
48. Sinajs D, Norris SJ, Weinstock GM. Genetic diversity in *Treponema pallidum*: Implications for pathogenesis, evolution and molecular diagnostics of syphilis and yaws. Infect Genet Evolu 2012;12:191-202.
49. Rawstron SA, Vetrano J, Tannis G, Bromberg K. Congenital syphilis: Detection of *Treponema pallidum* in stillbirths. Clin Infect Dis 1997;24:24-7.
50. Phelps RG, Knispel J, Schuman Tu E, Cernainu G, Saruk M. Immunoperoxidase technique for detecting spirochetes in tissue sections: Comparison with other methods. Int J Derma 2000;39:609-13.
51. Lee WS, Lee MG, Chung KY, Lee JB. Detection of *Treponema pallidum* in tissue: A comparative study of the avidin-biotin-peroxidase complex, indirect immunoperoxidase, FTA-ABS, complement techniques and the darkfield method. Yonsei Med J 1991;32:335-41.
52. Hoang MP, HighWA, Molberg KH. Secondary syphilis: A histologic and immunohistochemical evaluation. J Cutan Pathol 2004;31:595-9.