

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 FCCM⁴,
Gayatri C Jayaraman PhD MPH⁵, Kamran Kadkhoda PhD FCCM^{6,7}

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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

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

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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 immunohistochemique 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*.

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

¹National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba; ²BC Public Health Microbiology and Reference Laboratory;

³Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia; ⁴McMaster University, Hamilton;

⁵Centre for Communicable Diseases and Infection Control, Public Health Agency of Canada, Ottawa, Ontario; ⁶Cadham Provincial Laboratory;

⁷Department 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



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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 <i>Borrelia</i> 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 lose 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 $1500 \times g$ 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 $40\times$ objective ($400\times$ magnification) and any suspicious fluorescent objects should be verified by the oil immersion $100\times$ objective ($1000\times$ 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 *polA*, *tp47*, *bmp* and *tmpA* (33-36). One study comparing *polA*, *bmp* and *tp47* 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*, *tp47*, *rpsA* (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

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