Development of a Real-Time PCR Assay To Detect *Treponema pallidum* in Clinical Specimens and Assessment of the Assay’s Performance by Comparison with Serological Testing

David E. Leslie,* Franca Azzato, Theo Karapanagiotidis, Jennie Leydon, and Janet Fyfe

*Victorian Infectious Diseases Reference Laboratory, 10 Wreckyn St., North Melbourne, Victoria 3051, Australia*

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The incidence of infectious syphilis in men who have sex with men and human immunodeficiency virus-infected patients has increased steadily in Victoria, Australia, since 2002. A TaqMan real-time PCR assay targeting the *polA* gene of *Treponema pallidum* (TpPCR) was developed. The analytical sensitivity of the assay was estimated to be 1.75 target copies per reaction. Initially, the assay was used to test a variety of specimens (excluding blood) from 598 patients. Of the 660 tests performed, positive PCR results were obtained for 55 patients. TpPCR results were compared with serology results for 301 patients being investigated for early syphilis. Of these patients, 41 were positive by both TpPCR and serology, 246 were negative by both TpPCR and serology, 4 were TpPCR positive but negative by serology, and 10 were TpPCR negative but showed evidence of recent or active infection by serology. Directly compared with serology, TpPCR showed 95% agreement, with a sensitivity of 80.39% and a specificity of 98.40%. Potential factors leading to the discrepant results are discussed. Concurrent serology on 21 patients with TpPCR-positive primary syphilitic lesions demonstrated that a panel of current syphilis serological tests has high sensitivity for the detection of early syphilis. We found that TpPCR is a useful addition to serology for the diagnosis of infectious syphilis. Direct comparison with other *T. pallidum* PCR assays will be required to fully assess the limitations of the assay.

Since 2002, there has been a marked increase in the number of cases of locally acquired syphilis infection in Victoria, Australia (2). Most cases were detected in men who have sex with men, with a disproportionate number of cases in human immunodeficiency virus (HIV)-infected patients (3).

Clinical suspicion, and recognition of the symptoms and signs of syphilis, supported by serology is the mainstay of syphilis diagnosis, as direct detection methods such as dark-field microscopy and direct immunofluorescence are relatively insensitive, require fresh high-quality specimens, and are unsuitable for use on specimens from mucosal sites or if superinfection is present (4). Serology may be problematic in the early stages of primary syphilis, as rapid plasma reagin assay (RPR) responses may take some time to develop (4), particularly in HIV-infected patients, and few laboratories in Australia routinely use immunoglobulin M (IgM) assays.

The Victorian Infectious Diseases Reference Laboratory (VIDRL) acts as the state reference laboratory for syphilis serology, confirming positive results from other laboratories and offering less commonly used tests, such as IgM assays. The laboratory also provides primary diagnostic services to a number of Melbourne sexually transmitted infection (STI) clinics and receives large numbers of specimens for culture or PCR for other agents of STI, allowing easy access to specimens for the development and assessment of novel STI detection assays. Our aim was to develop a robust, sensitive, and specific real-time PCR assay to directly detect the presence of pathogenic *Treponema pallidum* in swabs and biopsy specimens from genital and mucosal ulcers, placental specimens, and cerebrospinal fluid. At the time of writing of this paper, no comparable assay for the direct detection of *T. pallidum* was available; an initial assessment of assay performance was done by comparison of PCR results with syphilis serology results where adequate serological follow-up had occurred.

**MATERIALS AND METHODS**

Population and specimens tested. Specimens tested by a TaqMan real-time PCR assay targeting the *polA* gene of *Treponema pallidum* (TpPCR) were referred to the VIDRL between February 2004 and December 2005. Initially, 174 genital specimens of various types sourced from Melbourne clinics with a high caseload of men who have sex with men, HIV patients, and other patients with high rates of STI were retrospectively blind tested by TpPCR. These specimens were sent by the attending physician for microscopy and bacterial culture, *Chlamydia trachomatis* PCR, *Neisseria gonorrhoeae* PCR, or herpes simplex virus (HSV) PCR. During this phase of the study, there were three positive TpPCR results. These early positive results were discussed with the physicians, and evidence of recent infection was confirmed by serology in all three cases. Once clinicians and laboratories became aware that a TpPCR assay was being trialed, specimens were referred specifically for TpPCR, including specimens for the investigation of congenital infections and neurosyphilis. The specimen types tested are shown in Table 2. Blood samples were not accepted for testing by TpPCR. Lesion swabs collected by the attending doctor were transported to the laboratory in either bacterial or viral transport medium. Cerebrospinal fluid specimens were sent fresh, and tissue specimens were sent either fresh or paraffin embedded in the case of two placental biopsy specimens.

Specimens sent for TpPCR were often received with concurrent requests for either culture or PCR for common agents of STI. A serum specimen for syphilis serology was not automatically referred to the laboratory with TpPCR requests, and in some cases, serology may have been performed by another laboratory; however, syphilis serology was concurrently or subsequently referred to the VIDRL for roughly half of all patients tested by TpPCR. Patient HIV status was
either known or determinable based on other HIV-related test results. Crude test and patient numbers by sex and HIV status are shown in Table 1.

**DNA extraction.** DNA was extracted using the Roche Diagnostics (Basel, Switzerland) MagNa Pure LC DNA isolation kit III (fungi and bacteria) according to the manufacturer's instructions.

**Assay design.** A TaqMan real-time PCR assay (minor groove binder [MGB] probe) was designed using the Primer Express software program (Applied Biosystems, Foster City, CA) targeting a 67-bp sequence within the polA gene of *T. pallidum*. This sequence (nucleotides 2001 to 2067) (GenBank accession no. TPU57757) is within the region amplified by primers F1 and R1 described previously by Liu et al. (5). A BLAST search performed using this 67-bp fragment indicated that apart from the *T. pallidum* polA gene, there were no similar sequences in the GenBank database. Primers were synthesized by Geneworks (GeneWorks Pty. Ltd., Hindmarsh, Australia), and the TaqMan MGB probe was synthesized by Applied Biosystems. The probe was labeled with the fluorescent dye 6-carboxyfluorescein at the 5' end and a nonfluorescent quencher at the 3' end (Applied Biosystems) (forward primer SyphTF [5'-AGG AGC GTG TCA TTC AGG ATC GCC CAT A-3'], reverse primer SyphTR [5'-GTG AGC GTC TCA TCA TTC -3'], and MGB probe SyphTP [6-carboxyfluorescein–ATG CAC CAG CAA A-3'–MGB nonfluorescent quencher]).

Real-time PCR mixtures contained template DNA, 0.9 μM concentrations of each primer, a 0.25 μM concentration of the probe, Absolute QPCR ROX (500 nM) mix (ABGene, Epsom, United Kingdom), and TaqMan exonous internal positive control (Applied Biosystems) in a total volume of 25 μl in a 96-well plate.

**Amplification and detection.** Amplification and detection were performed with the ABI Prism 7000 sequence detection system (Applied Biosystems) using the following program: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Controls.** A positive control was prepared by PCR amplifying a fragment within polA using primers F1 and R1 (5). Five microliters of a 1/10 dilution of this fragment gave a cycle threshold (Ct) of approximately 22 for each run. A no-template negative control was also included in each run, as was a reagent-only control. The TaqMan exonous internal positive control DNA and mix present in each well constituted the internal positive control for inhibition. Specimens and controls were tested in duplicate.

To determine the sensitivity of the assay, a suspension of *T. pallidum* strain Nichols that was previously used in the *T. pallidum* immobilization assay (kindly supplied by Serge Nesteroff, ICPMR, Westmead, Australia) containing 3.5 × 10^7 organisms per ml was serially diluted in phosphate-buffered saline, and 100-μl aliquots of the 1/10 (3.5 × 10^6 organisms) to 1/10^6 (three to four organisms) dilutions were applied to charcoal swabs. DNA was extracted as described above. This showed a detection limit of 35 organisms per swab (1.75 target copies per reaction) at a Ct of 38.4.

Using the above-mentioned information, and after an initial analysis of TpPCR results versus serology, an assay cutoff at cycle 38 was determined, and duplicate samples with a mean Ct of >38 were categorized as negative.

The performance of the TpPCR was assessed against serology by comparing results from a subset of 301 specimens of various types from 301 patients. Only the first TpPCR specimen submitted from each patient was included. Specimen types are shown in Table 2. For this part of the study, all specimens came from patients undergoing investigation for infectious (primary or secondary) syphilis who had a concurrent or subsequent syphilis serology requested. In many cases, results from prior serology testing were also available for comparison.

**Serology testing.** The syphilis serology tests used included RPR (Macro-Vue RPR card; Becton Dickinson, Sparks, MD), *T. pallidum* particle agglutination (TPPA) (Serodia TPPA; Fujirebio, Tokyo, Japan), recombinant total antibody enzyme immunoassay (EIA) (rEIA) (Treponostika TP recombinant; bio-Merieux, Boxtel, The Netherlands), and whole-cell-lysate IgM EIA (Mercia Syphilis IgM; Microgen Bioproducts, Camberley, Surrey, United Kingdom). All tests were performed according to the manufacturer’s instructions and are subject to three regular quality assurance programs per year.

All sera were routinely tested using RPR, TPPA, and rEIA. Sera collected concurrently from patients with positive TpPCR results were also tested for IgM by EIA, as were sera from other patients, regardless of the PCR result guided by direct requests for the assay, clinical notes on test requests suggesting the possibility of recent infection or exposure, or prior serology status.

Sera were considered to show evidence of recent infection if a concurrent serum specimen was positive or low positive by rEIA and TPPA and the IgM EIA was positive, regardless of the RPR result (in many cases, seroconversion from negative serology within the previous year could also be demonstrated), or a prior serum within the last 12 months was positive by rEIA/TPPA, indicating prior infection, but a concurrent serum sample showed a fourfold or greater rise in RPR with or without the presence of IgM.

Sera were considered not to show evidence of recent infection if all concurrent or subsequent (within 12 months) rEIA, TPPA, and IgM EIA results were negative or if serum tested within the last 12 months was positive by EIA/TPPA, indicating prior infection, but a concurrent IgM EIA was negative and RPR remained negative or showed no increase in titer.

**RESULTS**

Of the 660 specimens from 590 patients tested, positive TpPCR results were obtained for 55 specimens from 51 patients (Table 1). HIV-infected males had the highest rate of positive results. Eight patients had both positive and negative PCR results recorded at differing times and/or specimen sites. Results by specimen site tested are shown in Table 2. The only positive result from a female patient was from placenta and membranes from a hospital in Alice Springs, Northern Territory, Australia, following a fetal death in utero with suggestive changes of syphilis infection seen on histopathological examination.

A number of other organisms, including *Staphylococcus aureus*, beta-hemolytic streptococci, and HSV types 1 and 2, were
detected in specimens that were both positive and negative by TpPCR.

Of the 301 patients with adequate serological follow-up, the assay showed 95% agreement, with a sensitivity of 80.39% and specificity of 98.40%, compared with serology for the detection of early syphilis (Table 3).

Discrepant results included four patients (all HIV negative) with positive PCR results from penile lesions but no serological evidence of recent infection: all of these patients had mean $C_F$ values of $>35$. Apart from the detection of HSV type 2 in two of these patients, no further clinical information was available.

Ten patients (six HIV infected) had a negative PCR result (from nine genital lesion swabs and one mouth ulcer swab) but evidence of recent infection by serology. In one patient, a swab collected from the same site 7 days later was PCR positive. In the patient with the mouth ulcer, a concurrent anal swab that was not tested by TpPCR tested positive for C. trachomatis and N. gonorrhoeae.

Excluding patients with reinfections or clinical notes indicating nonprimary syphilis, 21 patients with a positive PCR result had all available serology tests performed on a concurrent serum sample. Of the 20 patients with more than one positive serology test result, TPPA was positive for all 20 of these patients, rEIA was positive for 18 patients and low positive for 2 patients, and IgM EIA was positive for 19 patients and low positive for 1 patient. RPR was negative for 2 patients, positive with a reciprocal titer of 1 (R1) to R2 for 4 patients, and positive with a titer of R4 to R256 for 14 patients. The other patient had an equivocal IgM EIA result only, but a serum specimen collected 10 days later was low positive by both IgM EIA and rEIA.

**DISCUSSION**

Studies of this type have several problems, the major one being the lack of a “gold standard” for the direct detection of T. pallidum with which to compare the PCR assay. Serological confirmation may be delayed, absent, or difficult to interpret in cases of potential reinfection or reactivation of disease, and some patients, such as HIV-infected patients with low CD4 counts, may have atypical responses (4).

Discrepant results were obtained for 14 patients; those that were TpPCR positive but negative by serology all had $C_F$ values close to the cutoff at cycle 38. These results may represent the detection of low levels of T. pallidum or nonviable organisms that failed to initiate an invasive infection in the patient or may represent false-positive results due to low-level assay contamination. It was not known if any of these patients were receiving antibiotics at the time of sampling.

For the PCR-negative but serology-positive patients, the most likely explanation for the lack of correlation is incorrect timing or site of sampling. One patient with seroconversion and a positive PCR from a penile ulcer with a mean $C_F$ of 34.6 showed a weaker positive result ($C_F$ of 37.7) when the lesion was resampled 7 days later. This suggests that only small amounts of T. pallidum DNA may remain in healing chancres, nearing or exceeding the detection limit of the assay. To date, there have been few other published studies that compared syphilis PCR assays with serology results. A study by Orle et al. (6) in 1995 used a T. pallidum 47-kDa integral membrane lipoprotein gene target in a multiplex AMPLICOR PCR format (Roche Molecular Systems) and confirmed results with a second PCR assay targeting the basic membrane protein (bmp) gene. Correlation with RPR or VDRL serology was 88% in a population of 296 American STI clinic patients; however, no T. pallidum-specific serology was performed. In 2001, Bruisten et al. (1) used a bmp gene target in a nested gel-based PCR format and compared results against RPR, TPHA, and fluorescent treponemal antibody data in a population of 364 Dutch STI clinic patients. They found a correlation of 96%; however, syphilis infection was uncommon in the population studied, with 12 cases detected by PCR, 7 cases detected by serology, and only 3 cases detected by both PCR and serology. In 2003, Palmer et al. (7) used a gel-based PCR assay to detect the T. pallidum 47-kDa integral membrane lipoprotein gene in a population of 98 STI clinic patients in the United Kingdom and compared the results with serology using a combination of RPR, TPPA/TPHA, and IgG and IgM EIAs. Those authors estimated the sensitivity and specificity of the PCR for the detection of primary syphilis to be 94.7% and 98.6%, respectively, and estimated the sensitivity and specificity of the PCR for the detection of secondary syphilis to be 80% and 98.6% compared with serology. Dark-ground microscopy was positive for only 2 of 10 patients with primary syphilis and none of 3 patients with secondary syphilis. Those authors described three PCR-negative but serology-positive patients and proposed that those results were due to sampling error or inadequate sensitivity of the PCR assay, and those authors also described one PCR-positive but serology-negative result for an HIV patient with a low CD4 count who also received antibiotics 6 days after the positive PCR swab was taken and postulated that the combination of a blunted immune response and early treatment was the likely explanation for the lack of a serological response.

More targeted studies including more detailed clinical information, repeat sampling, and direct comparison of other PCR techniques will be required before a more precise assessment of sensitivity and specificity can be estimated. However, overall, there was 95% agreement between PCR and serology results in the investigation of early syphilis. This study also highlights the importance of laboratories performing adequate serological testing guided by clinical information, as laboratories that screen with RPR alone will fail to detect a small number of cases of infectious syphilis. This study also demonstrates the high sensitivity of modern T. pallidum-specific assays (TPPA, IgM EIA, and rEIA) in early syphilis, and the widely quoted (4) 1- to 4-week “window period” after the appearance of the chancre, but before the development of antibodies, may be reduced in laboratories using a range of
modern commercial syphilis serology assays. Whether the use of the single-copy polA gene target will provide adequate sensitivity for the use of this assay in the later stages of syphilis, where low levels of *T. pallidum* are widely distributed throughout the body, will be difficult to assess, as late syphilis is a rare disease in Australia at present.

In initial and ongoing studies, a few positive PCR results were obtained for specimens submitted only for other tests such as herpesvirus PCR or bacterial culture, and syphilis serology was not concurrently requested for these patients. This would imply that the clinical diagnosis of syphilitic lesions is not always a simple matter, particularly in HIV-infected patients and in patients with syphilitic lesions superinfected with other organisms. We believe that the *T. pallidum* PCR will be a valuable addition to serology for the diagnosis of early syphilis and will be useful for the confirmation of other diagnostic methods such as histopathology in late and congenital syphilis.

REFERENCES

AUTHOR’S CORRECTION

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