Use of *Treponema pallidum* PCR in Testing of Ulcers for Diagnosis of Primary Syphilis

Angèle Gayet-Ageron, Patrice Sednaoui, Stephan Lautenschlager, Tristan Ferry, Laurence Toutous-Trellu, Matthias Cavassini, Fatiama Yassir, Begona Martinez de Tejada, Stephane Emonet, Christophe Combescure, Jacques Schrenzel, and Thomas Perneger

*Treponema pallidum* PCR (Tp-PCR) has been noted as a valid method for diagnosing syphilis. We compared Tp-PCR to a combination of darkfield microscopy (DFM), the reference method, and serologic testing in a cohort of 273 patients from France and Switzerland and found the diagnostic accuracy of Tp-PCR was higher than that for DFM.

Incidence of syphilis, caused by *Treponema pallidum*, has increased steadily worldwide since the early 2000s, especially in at-risk populations (1). The US Centers for Disease Control and Prevention (CDC) recently updated the definitions for confirmed cases of primary and secondary syphilis and now considers *Treponema pallidum* PCR (Tp-PCR) to be a valid diagnostic method along with darkfield microscopy (DFM) (2), which is still considered the reference test (although it remains imperfect) (3). In diagnosis of sexually transmitted ulcerative disease, a positive DFM result confirms syphilis because other *T. pallidum* subspecies are not sexually transmitted and have a different geographic distribution. However, the meaning of a negative DFM result is more uncertain. Samples from up to 20% of case-patients with syphilis may show negative DFM results when the test is performed by technicians who are not fully trained or when it is performed in suboptimal conditions (3). Tp-PCR is clinically useful for testing of ulcers or skin lesions in areas where syphilis prevalence is high (4), but uncertainties remain because of the variability in the reference tests used in the different diagnostic studies. Moreover, the risk for misclassification by DFM diminishes the apparent value of Tp-PCR when DFM is the reference test because samples from syphilis patients that yield a negative DFM result, but a positive Tp-PCR result, are currently considered false-positive.

We conducted a multicenter study in France and Switzerland to evaluate the accuracy of Tp-PCR compared with DFM and serologic testing. To resolve the difficulty of assessing a new diagnostic test against an imperfect standard, in addition to the standard DFM diagnostics, we used an enhanced definition for the diagnosis of syphilis that combines clinical information with DFM, serologic testing, or both, to enable a fair assessment to be made of the diagnostic performance of Tp-PCR.

The Study

We conducted a multicenter, prospective, observational study during September 2011–September 2013 in 5 centers in Switzerland and France (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/1/14-0790-Techapp.pdf). All patients who had a genital, anal, or oral ulcer suggestive of syphilis after having at-risk sexual intercourse were invited to participate in the study. We used 3 definitions that would indicate a diagnosis of syphilis: 1) positive DFM results (5); 2) a combination of nontreponemal and/or treponemal tests as recommended by CDC (2) (if possible, samples that had negative results on a first nontreponemal assay underwent a second test to identify seroconversion [6]); and 3) an enhanced definition combining clinical information suggestive of syphilis and results from DFM and serologic testing. The diagnosis of syphilis was established by positive DFM results or negative DFM results combined with positive serologic tests as defined by the second definition, plus a clinical outcome and a drop in nontreponemal titers in response to treatment.

Clinicians collected ulcer specimens in a standardized manner. All samples were then sent to the bacteriology laboratory at the University of Geneva Hospitals, where all Tp-PCR testing was performed by using a previously published protocol (7) and interpreted without knowledge of the patient’s clinical or serologic status.

We recruited 273 patients from the 5 centers: 140 from Paris, France; 59 from Lyon, France; 40 from Geneva, Switzerland; 17 from Lausanne, Switzerland; and 17 from Zurich, Switzerland. Patients had a mean age of 39.0 years (SD 12.2); most (252, 92.3%) were men. Mean delay from ulcer appearance to date of first medical visit was 20.4 days.

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1Preliminary results from this study were presented at the 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy, September 10–13, 2013, Denver, Colorado, USA.
(SD 33.9; n = 132). Most patients were men who have sex with men (n = 185 [71.4%]). Ulcer localization was genital (n = 148, 54.2%), anorectal (n = 98, 35.9%), or oral (n = 27, 9.9%). HIV status was known for 226 patients (82.8%); 53 were HIV positive, and 36 were receiving antiretroviral drug therapy. Nine patients received an initial HIV diagnosis at the time of the diagnostic work-up for syphilis.

DFM results were assessed for 170 patients (62.3%); 32 had positive results (18.8%). Results for 43 Tp-PCR specimens were positive; 13 of these were from patients who had negative DFM results. The proportion of positive DFM-positive Tp-PCR results was significantly higher for the 2 centers where DFM was performed only occasionally (6/15 [40.0%]) than for centers who performed DFM more often (7/155 [4.5%]; p<0.001). The diagnostic performance of Tp-PCR against DFM was high (Table 1), and agreement between the 2 tests was substantial.

Specimens from 255 patients underwent serologic testing; 88 patients had positive results, and 16 patients had undetermined results. Results for Tp-PCR were less sensitive and had a lower negative predictive value when serologic tests results were used as reference than when DFM results were used as reference (Table 1). Under the enhanced definition, however, 16 patients who had negative DFM results were considered to have syphilis, and Tp-PCR provided higher specificity and positive predictive value when compared with this definition than when compared to either DFM or serologic test results alone (Table 1). When DFM was assessed against Tp-PCR and the enhanced definition (Table 2), DFM sensitivities were consistently lower. Additional results are shown in the online Technical Appendix.

Conclusions
Our results demonstrate that Tp-PCR has a high degree of accuracy for the definitive diagnosis of primary syphilis from lesion exudate or tissue. As expected, the clinical value of this test appeared sensitive to the choice of reference test but was hampered by misclassification errors from DFM. By definition, any discrepancy between Tp-PCR and DFM results has been considered primarily an error in Tp-PCR. However, this assumption may not always be accurate.

The reliability of DFM in our study was strongly associated with routine performance. We classified cases with negative DFM results, positive serologic results, and a clinical picture evocative of syphilis as false negatives of the DFM. When we used this definition as a reference, the diagnostic performance of Tp-PCR appeared higher, indicating that Tp-PCR has a high clinical usefulness either for confirming or for ruling out a suspicion of syphilis.

The strengths of our study are its prospective and multicenter design and the performance of Tp-PCR in a unique laboratory. The study sample was also representative of patients who may benefit from Tp-PCR in the future. The main limitation was the lack of a standard protocol for serologic testing, which could have affected the validity of some analyses. However, we attempted to minimize intercenter variability by using a blind assessment of all serologic assays by 2 experts.

Our results concur with those of Grange et al., who reported that Tp-PCR provides better sensitivity/specificity than DFM when compared with clinical suspicion of syphilis (8). Similarly, Heymans et al. estimated 87.0% sensitivity and 93.1% specificity of Tp-PCR compared with DFM (9).

| Table 1. Summary of the various indices of performance of Tp-PCR compared with DFM, serologic testing, or an enhanced definition for diagnosis of primary syphilis* |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Reference testing | Sensitivity (95% CI) | Specificity (95% CI) | Likelihood ratio (95% CI) | Post-test probability (95% CI) |
| | | | Positive | Negative | PPV | NPV |
| DFM | 93.8% (79.2%–99.2%) | 90.6% (84.4%–94.9%) | 9.95 | 0.07 | 0.74 | 69.8% (53.9%–82.8%) | 1.6% (0.2%–5.6%) |
| Enhanced definition | 74.8% (68.4%–81.5%) | 95.5% (89.1%–99.9%) | 6.84–21.77 | 0.16–0.35 | 0.64–0.82 | 78.7% (70.5%–86.8%) | 6.7% (4.3%–9.7%) |
| Serologic | 78.5% (72.3%–84.8%) | 93.4% (87.3%–98.5%) | 11.84 | 0.23 | 0.73 | 87.8% (79.6%–95.9%) | 11.0% (1.5%–20.8%) |
| n = 239 | 93.4% (87.3%–98.5%) | 93.4% (87.3%–98.5%) | 11.84 | 0.23 | 0.73 | 87.8% (79.6%–95.9%) | 11.0% (1.5%–20.8%) |
| Enhanced | 74.8% (68.4%–81.5%) | 95.5% (89.1%–99.9%) | 6.84–21.77 | 0.16–0.35 | 0.64–0.82 | 78.7% (70.5%–86.8%) | 6.7% (4.3%–9.7%) |

*Tp-PCR, Treponema pallidum PCR; DFM, darkfield microscopy; PPV, positive predictive value; NVP, negative predictive value.

Table 2. Summary of the various indices of performance of DFM compared with Tp-PCR or an enhanced definition for diagnosis of primary syphilis*

<table>
<thead>
<tr>
<th>Reference testing, n = 170</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Likelihood ratios (95% CI)</th>
<th>Post-test probability (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Tp-PCR</td>
<td>66.7% (59.3%–73.8%)</td>
<td>96.9% (91.3%–99.8%)</td>
<td>44.30</td>
<td>0.31</td>
</tr>
<tr>
<td>Enhanced definition</td>
<td>66.7% (59.3%–73.8%)</td>
<td>96.9% (91.3%–99.8%)</td>
<td>44.30</td>
<td>0.31</td>
</tr>
</tbody>
</table>

*Tp-PCR, Treponema pallidum PCR; DFM, darkfield microscopy; PPV, positive predictive value; NVP, negative predictive value.
Currently, DFM is less often used in routine testing than it has been in the past (10). A survey of infectious diseases specialists found that 56% have systematically performed a rapid plasma reagin test before starting treatment for syphilis (10). Only 18% repeated the test if results were negative (10), and just 2% applied direct syndromic management (11). These numbers demonstrate a lack of consensus in the decision-making process used by experts and suggest that applying the guidelines for diagnosis of syphilis is difficult in daily practice. Moreover, although serologic testing can provide a background value for the interpretation of future tests and the assessment of treatment response, these results are often noninformative in the early phase of the infection, when up to 30% of tests return false-negative results (12).

In summary, our results confirm that using Tp-PCR as the reference diagnostic test for early-phase syphilis may be reasonable (2). Several arguments weigh in favor of Tp-PCR. First, Tp-PCR was more accurate than DFM when assessed against the enhanced definition in our study. Second, high-quality readings of DFM are difficult to obtain (3), especially when the test is not routinely performed. Finally, the Tp-PCR test relies less on human expertise than DFM, which may make Tp-PCR results more reproducible and testing less costly if it is performed on a routine basis.

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Dr. Gayet-Ageron is a medical doctor and researcher in the Division of Clinical Epidemiology and Infection Control Program of the University of Geneva Hospitals and Faculty of Medicine, Geneva, Switzerland. Her primary research interest is epidemiology and clinical research in the field of infectious diseases.

References

Address for correspondence: Angele Gayet-Ageron, Division of Clinical Epidemiology, University of Geneva Hospitals, 6 rue Gabrielle Perret-Gentil, 1211 Geneva 14, Switzerland; email: angele.gayet-ageron@hcuge.ch

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Technical Appendix

Methods

Study design, setting and study population

We conducted a multicenter, prospective, observational study between September 2011 and September 2013 in five European centers: a sexually-transmitted diseases (STD) outpatient clinic in Paris, France; the STD and infectious diseases outpatient clinics at a tertiary hospital in Lyon, France; the STD and gynecology outpatient clinics at a tertiary hospital in Geneva, Switzerland; the dermatology and infectious diseases outpatient clinics at a tertiary hospital in Lausanne, Switzerland; and the dermato-venereology outpatient clinic at a tertiary hospital in Zurich, Switzerland.

The study protocol was approved by the local institutional review boards and was exempted from approval in France since it was considered as non-interventional. All patients provided written consent before inclusion.

Diagnosis of syphilis

We used three case definitions of syphilis as described in the main paper. The confirmed case was based on dark-field microscopy (DFM) routinely performed by the same two investigators (PS and SL) in two centers (Paris and Zurich), but was occasionally performed by the physicians in charge of patients in two others (Geneva and Lausanne). In the two centers where DFM was performed occasionally, the current study served as a way to maintain the knowledge of young physicians regarding the performance of DFM.

The probable syphilis case definition was based on the combination of the Venereal Diseases Research Laboratory test (VDRL) or rapid plasma reagin (RPR) alone or combined with a microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP), a fluorescent treponemal antibody-absorbed test [FTA-ABS], or enzyme immune-assay (EIA). The sequence
of nontreponemal and treponemal assays was left to the physician’s discretion. Syphilis was diagnosed if VDRL or RPR was reactive and combined with positive MHA-TP or FTA-ABS in the absence of syphilis history. Positive EIA led to a diagnosis of syphilis if combined with reactive VDRL/RPR plus positive MHA-TP or FTA-ABS. Interpretation of serology was performed by two independent experts (PS and SL) and disagreement was resolved by consensus.

Other diagnoses for STD ulcers

Other diagnostic tests could be ordered to diagnose a STD ulcer, such as herpes simplex culture, immunofluorescence or PCR, Chlamydia trachomatis PCR, Neisseria gonorrhoeae culture or PCR, and identification of Haemophilus ducreyi on culture.

DNA extraction from ulcer swabs and real-time Tp-PCR

Clinicians were instructed to collect ulcer specimens by first gently removing necrotic material or crusts from the lesions with sterile gauze, then gently expressing the clear exudate from the ulcer. The exudate was then adsorbed on Dacron swabs and sent directly to the bacteriology laboratory at the University of Geneva Hospitals in 3mL of universal transport medium (Copan International, Murrieta, CA, USA). Samples collected in French centers were frozen at −20°C and shipped on dry ice. All Tp-PCRs were performed in Geneva following a previously published protocol (1). The result of Tp-PCR (primary outcome) was expressed as a binary result (positive/negative). We performed three technical replicates for each clinical sample and obtained three cutoff positive cycle thresholds (C_T). Tp-PCR was considered positive if at least two of three C_T were below 40.

Variables collected in the case report form and follow-up

Sociodemographic data, medical history, and clinical data related to the current episode of STD ulcer were anonymously collected in a standardized case report form. Four centers (Geneva, Zurich, Lausanne, and Lyon) provided data on serologic follow-up (VDRL/RPR±MHA-TP) at 3-, 6-, or 12-month intervals after treatment. Treatment response was defined by a 4-fold decline in nontreponemal test titers (2).

Sample size estimation

Based on previous studies, 80% of patients with syphilis will have a positive Tp-PCR (3). Therefore, 61 cases of early syphilis were needed to obtain a total width of the 95% confidence interval (CI) of 20% (80% ± 10%) (4). We anticipated that 25% of eligible patients would have
syphilis (5,6), which led to a total of 260 patients, assuming 5% missing data. With 200 controls, we expected to estimate a specificity of 90% with a total width of 95% CI of 16% (90% ± 8%).

**Statistical analysis**

The study population was described using mean ± standard deviation (SD) or median (interquartile range [IQR]) for continuous variables, and frequencies and proportions for categorical variables. Comparisons of continuous variables were done using non-parametric Mann-Whitney tests. Comparisons of categorical variables were done using the Chi-square test.

The diagnostic performance of $Tp$-PCR was assessed with DFM as the reference test. Sensitivity, specificity of $Tp$-PCR, and post-test probabilities regarding syphilis diagnosis (positive predictive value and one minus negative predictive value) were computed together with 95% CIs obtained by the Clopper-Pearson method (7). We calculated also positive and negative likelihood ratios (8). Agreement between $Tp$-PCR and DFM was assessed by kappa coefficients (with exact 95% CIs) and interpreted following the Landis and Koch scale (9). The diagnostic performance of $Tp$-PCR was then assessed with a reference test combining nontreponemal and treponemal tests and agreement was assessed. We also assessed also the diagnostic performance of DFM with 95% CI against $Tp$-PCR and against the enhanced definition.

All statistics were accompanied by their 95% CI. Statistical significance was defined as $p < 0.05$ (two-sided). All analyses were performed using Stata intercooled 13.0 (STATA Corp., College Station, TX, USA).

**Results**

**Complementary diagnoses for ulcerative diseases**

Nine patients were concomitantly diagnosed with new HIV infection and seven had negative VDRL/RPR and TPHA. For the two patients with positive serology, syphilis was retained. Ulcers were attributed to HIV for three cases and the rest was attributed to single or a combination of other pathogens ($Chlamydia trachomatis$, $n = 4$; herpes simplex virus type 2, $n = 3$; $Neisseria gonorrhoeae$, $n = 1$).

Among the 32 positive DFM, six were co-infected (18.8%) with other pathogens ($Chlamydia trachomatis$, $n = 2$; herpes simplex virus type 2, $n = 1$; $Neisseria gonorrhoeae$, $n = 1$; $Haemophilus parainfluenzae$, $n = 1$; M or group C $Streptococcus$, $n = 1$). Among the 138 patients
with negative DFM, the diagnosis was herpes simplex virus type 1 or 2 (n = 21; 15.2%), infections with *Chlamydia trachomatis* (n = 13; 9.4%), or *Neisseria gonorrhoeae* (n = 7; 5.1%), and polyinfection (n = 10; 7.2%). Twelve (8.7%) patients with negative DFM were nonetheless treated for syphilis. Among the 88 positive serologic cases, 10 (11.4%) were co-infected with another pathogen. Other diagnoses were herpes simplex virus type 1 or 2 (n = 25; 16.5%), *Chlamydia trachomatis* (n = 9; 6.0%), or *Neisseria gonorrhoeae* (n = 8; 5.3%), and polyinfection (n = 5; 3.3%).

**Diagnostic performance of Tp-PCR with DFM as reference test**

Among positive *Tp*-PCR, $C_T$ values were similar between patients with positive and negative DFM (median 33.3 versus 33.0, respectively; $p = 0.89$).

**Diagnostic performance of Tp-PCR with serology as reference test**

A total of 255 patients were tested by serology (Appendix 3). The most frequent combinations were VDRL/RPR plus MHA-TP (n = 137 [53.7%]), VDRL/RPR plus MHA-TP and/or FTA-ABS (n = 46 [18.0%]), reactive EIA (n = 45 [17.6%]) confirmed by VDRL/RPR plus FTA-ABS and/or MHA-TP, and other combinations (n = 27 [10.6%]). The two experts had high agreement in the interpretation of serology results (kappa, 0.88; 95% CI, 0.83–0.93).

**Follow-up of patients treated for syphilis**

A total of 78 patients were considered by clinicians as having syphilis by either positive DFM or positive serology and were treated. Of these, 62 had a positive *Tp*-PCR (79.5%). Of the 40 patients (51.3%) with serologic follow-up, 87.5% (n = 35) showed a 4-fold decrease in VDRL or RPR titers. Among the 13 patients with negative DFM and positive *Tp*-PCR, seven were treated by intramuscular benzathine-penicillin injections (53.8%) due to a high clinical presumption of syphilis, and five (71.4%) had a 4-fold decline in nontreponemal test titers at 3 months or later signing treatment response.

**References**


