Getting the measure of syphilis: qPCR to better understand early infection

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ABSTRACT

Objectives Until recently, PCR had been used to detect but not quantify Treponema pallidum. To understand infection kinetics of this uncultivable organism, a real-time PCR assay was developed to quantify 47 kDa membrane lipoprotein gene DNA (tpp47).

Methods Assay specificity was determined against DNA from humans, skin organisms and sexually transmitted pathogens. tpp47 DNA (Nichols strain) was used to construct a standard curve for T pallidum quantification. Blood and ulcer samples were obtained from 99 patients being investigated or screened for syphilis and tpp47 was quantified.

Results The assay was specific, not cross-reactive with other organisms tested and sensitive, with a detection limit of a single copy of tpp47 DNA. For ulcer samples, the assay was 100% sensitive and 97.14% specific. Sensitivity fell to 34.1% for blood samples but specificity remained high (100%). tpp47 DNA was more commonly detected, and at a higher copy number, in blood of patients with secondary infection (sensitivity 57.89%) compared with primary infection. Quantity of tpp47 DNA was higher in primary infection ulcers, especially in HIV-1-positive patients, than in ulcers persisting into secondary disease.

Conclusions Quantifying T pallidum provides insight into syphilis infection kinetics: Ulcers of primary disease in HIV-1-positive patients are perhaps more infectious and the presence and load of T pallidum bacteraemia is variable, with a peak in the secondary stage. Quantitative PCR has the potential to map T pallidum infection and to highlight the impact of HIV on syphilis.

INTRODUCTION

A better understanding of syphilis may be obtained by quantitatively mapping the course of infection. However, the causative organism, Treponema pallidum subspecies pallidum (T pallidum), cannot be easily cultured, so detection relies on direct visualisation, molecular detection and, rarely, rabbit infectivity testing. None of these methods is ideal for studying T pallidum pathogenesis or infection kinetics. Dark ground microscopy (DGM) to count organisms in ulcer exudate or cerebrospinal fluid is highly operator dependent and cannot be used for blood.1 Rabbit infectivity testing, often described as the gold standard for diagnosing syphilis,2 is highly sensitive but impractical. The non-specific anti-cardiolipin-based tests, for example, rapid plasma reagin (RPR), can be used as a surrogate marker of T pallidum burden to stage infection and monitor treatment response but are prone to false-positive results and do not reflect the kinetics of the infection since they take months to resolve and may never become negative. Furthermore, HIV-1 infection, which can alter the course of T pallidum infection and response to treatment, is also known to have an impact on treponemal serology.3 4

Molecular detection of T pallidum DNA by PCR is now established for the diagnosis of syphilitic ulcers.5 6 These assays have been based on the detection of various T pallidum genes, including polA,7–9 flaA,10 tmpA11 and tpp47,12–14 which are highly conserved across T pallidum subspecies. Until recently, molecular studies were qualitative and little was known about the quantity of T pallidum in clinical samples during the course of syphilis.

To study T pallidum infection kinetics in humans, a real-time PCR assay has been developed to detect and quantify T pallidum tpp47 DNA in samples from patients with syphilis.

METHODS

Population and specimens

A prospective, cross-sectional study was conducted at St Mary’s Hospital, London, between July 2006 and January 2008. The study had research ethics approval and samples were collected after obtaining written consent. All patients being investigated for syphilis (including routine screens) were eligible. Investigation and treatment for sexually transmitted infections (STIs) were performed in accordance with national guidelines.15

Six millilitres of blood were collected into EDTA for T pallidum DNA detection and quantification from all participants. Samples collected between July and December 2006 were processed immediately and DNA was extracted as described below. Samples collected after this period were stored at −80°C until DNA was extracted. Ulcers were cleaned and bandaged with sterile water and gauze prior to sampling. After 1–2 min, the accumulated exudate was absorbed onto Sno Strips filter paper (Laboratoire Chauvin, Aubenas, France) held at the ulcer edge until dry. DGM was performed on genital ulcers according to clinical policy prior to the collection of study samples.

The Newmarket IgM/IgG enzyme immunoassay (EIA) (Alere, Stockport, UK) was used to screen for T pallidum infection. Fujirebo T pallidum particle agglutination (TPPA) (Mast Diagnostics, Bootle, UK) and Bio-kit (RPR) (Launch Diagnostics, Longfield, UK) were used to confirm and quantify the serological response, respectively.

Baseline and convalescent syphilis serology were documented in addition to STI results. Patients’ clinical-microbiological diagnoses were recorded as one of the following: herpes simplex...
virus (HSV), primary syphilis, secondary syphilis, other syphilis (comprising latent and symptomatic late disease), neurosyphilis and asymptomatic contacts of syphilis and non-specific genital ulceration. Case definitions are given in table 1.

Demographic data, sexual orientation and HIV-1 status, if known, were also recorded. Ninety-nine patients were recruited of whom 44 had a final clinical diagnosis of syphilis: 14 had primary syphilis, 19 secondary syphilis, 8 early latent syphilis and 5 neurosyphilis (all during the secondary stage). Of the 55 non-syphilis cases, 17 (31%) were contacts of patients with syphilis and 38 had non-syphilitic ulcers of which half were confirmed as genital herpes. The remaining 19 (35%) patients had no STI and were diagnosed with non-specific genital ulceration, which is consistent with studies examining the cause of genital ulceration.17 18

The study population is representative of the current UK syphilis epidemic.19 Ninety-seven (98%) of the patients were men, 88 (88.7%) were Caucasian with a median age of 36 years and just over half (52, 52.5%) were HIV positive. Neither of the two women were HIV positive (full data shown in online supplementary table 1).

Sample handling and DNA extraction

Aliquots of 400 μl of EDTA whole blood (WB) were either immediately treated with 60 μl of protease K (Qiagen, Crawley, UK) or frozen at −80°C for future processing. DNA was extracted using the Qiagen QIAamp DNA Mini kit (blood spin protocol) with the following modifications: 400 μl of sample and buffer AL were used; the first incubation was for 5 h or overnight; final elution was into 120 μl of buffer AE following 5 min incubation. Extraction was performed in a separate laboratory to the PCR and new aliquots of reagent were used for each batch.

Whole Sno Strips were placed in microcentrifuge tubes with 400 μl phosphate buffered saline and 20 μl protease K. DNA was extracted using the Qiagen QIAamp DNA Mini kit, buccal swab spin protocol with the above modifications. Quantitative PCR (qPCR) results for ulcer samples are presented as copies/strip.

### Table 1 Case definitions

| Stage/disease | Definition |
|---------------|------------|
| HSV           | Typical single or multiple genital ulcers from which HSV-1 or HSV-2 DNA is isolated using an in-house PCR. |
| Primary syphilis | Diagnosis requires the presence of a chancre on examination and microbiological confirmation of Treponema pallidum with any of the following: dark ground microscopy, PCR provided by the sexually transmitted bacteria reference laboratory, Health Protection Agency, London, or positive treponemal serology (as per national guidelines).16 Primary diagnoses to include dark-ground negative ulcers and initially negative serology, which becomes positive up to 3 months later with no sign of secondary disease. |
| Secondary syphilis | Defined as syphilis within the first 2 years of infection and characterised by both clinical and microbiological findings. Clinical findings include typical rash, condylomata lata, muco-cutaneous lesions, generalised lymphadenopathy, anterior uveitis, hepatitis and splenomegaly. Microbiological findings must include serological tests consistent with secondary disease and may, if available, include PCR of secondary lesions.16 If anogenital examination reveals persisting chancres, the case is still counted as secondary. |
| Other syphilis | Latent syphilis; Positive serological tests in the absence of any symptom or sign. Classified as early within the first 2 years of infection and late thereafter. Symptomatic late syphilis; Positive serology including a negative or low and unchanging RPR titre together with symptoms and clinical signs of cardiovascular or gummatous disease. Late disease with neurological involvement is classed as neurosyphilis. |
| Neurosyphilis | Any stage of disease with clinical neurological involvement (including meningitis, cranial nerve palsies, parenchymatous general paresis, tabes dorsalis), serological findings consistent with the stage of disease and a relevant CSF abnormality (as per national guidelines).16 NB: For analysis, cases with neurological involvement were counted as neurosyphilis not by stage. |
| Asymptomatic contact of syphilis | Patient reports sexual contact with a suspected or confirmed case of syphilis. Examination reveals no sign of disease and serological results (baseline, 6 weeks and 3 months after exposure) are not consistent with current infection. |
| Non-specific genital ulceration | Genital ulceration is present on examination, but no microbiological diagnosis is made. PCR for HSV is negative, DGM is negative, syphilis serology is not consistent with current infection and diagnostic T pallidum PCR (if performed) is negative. |

*Patients without neurological symptoms do not routinely undergo CSF examination at our centre.*

**Basic science**

Real-time PCR

A 178 bp segment of the 1377 bp T pallidum tpp47 gene (GenBank accession number M88769.1) was amplified using the following primers: forward 5′ CGAGGATAAAGATTAGG AACG3′, reverse 5′ ACGTGCGAAGAACACTATCCTCAG3′ (nucleotides 525–547 and 702–680, respectively). Primer sequences, product size and reaction conditions were optimised for sensitivity and reproducibility (data not shown). Each reaction contained 10 μl extracted DNA, 0.6 units LightCycler Uracil-DNA glycosylase (UDG) (Roche, Mannheim, Germany), 0.5 μm each of forward and reverse primers (Invitrogen, Paisley, UK), 4 μl FastStart Plus reaction mixture (Roche, Mannheim, Germany) (containing FastStart Taq DNA polymerase, reaction buffer, MgCl2, SYBR Green I dye and dNTP mix) and PCR-grade water to a final 20 μl volume.

Real-time PCR was performed using a Roche LightCycler 2, software version 4.0, with the following conditions: UDG activation at 40°C for 10 min, reversepreincubation at 95°C for 10 min and then 46 cycles of 95°C for 10 s, 60°C for 5 s, 72°C for 8 s and 81°C for 10 s (acquisition). Two no-template controls were
included in each experiment, and standard precautions to avoid contamination were employed.

Samples were run in duplicate and discordant results were repeated. Negative samples were diluted 1:10 and repeated if inhibition was suspected.

**Assay specificity**
During assay development, PCR product identity was confirmed using both agarose gel electrophoresis (2% agarose gel containing Tris-acetate-EDTA buffer, 0.6 mg ethidium bromide) and DNA sequencing using the Applied Biosystems dRhodamine kit and 3100 capillary sequencer. Using NCBI BLAST software, the 178 bp amplicon showed 100% sequence homology in comparison with published *T. pallidum* 47 kDa gene sequences.

Melting curve analysis was performed on all amplicons from clinical samples and compared with an in-run standard in order to confirm amplification of the correct target. Fluorescence emission in the 530 nm channel at a linear temperature transition rate of 0.1/°C/s from 60°C to 95°C was monitored with continuous acquisition. The melting temperature (Tm) of the correct 178 bp amplicon was 82.5°C.

In order to confirm non-cross-reactivity with commensal treponemes, the infective agents of other STIs, common skin organisms or human DNA, the assay was tested against DNA from these sources (see online supplementary table 2).

**Preparation of the standard curve and limit of assay detection**
*T. pallidum* (Nichols strain) bacteria were enumerated using DGM to give an initial concentration of 6 × 10^9 organisms/ml. DNA was extracted from 10 µl aliquots using Qiagen QIAamp DNA Mini kit as per the manufacturer’s protocol and eluted into 60 µl of molecular-grade water to produce a concentration of 10^6 *T. pallidum* genome equivalents/10 µl. Serial fivefold dilutions were made using molecular-grade water from 1/5 to 1/10^8.

DNA detection was unreliable beyond 1/10^6 dilution (CT 38.04), thus 1 copy/10 µl was determined to be the lowest limit of reliable detection. An example of an in-run standard curve used for *T. pallidum* DNA quantification in clinical samples is shown in the online supplementary data.

In order to be sure that the standard curve was biologically relevant, detection of *T. pallidum* DNA in WB and water were compared. Serial 10-fold dilutions of *T. pallidum* DNA were added to syphilis-sonerogative WB, quantified and found to be similar to dilutions in molecular-grade water described above (see online supplementary table 3).

**Bacteria and DNA**
*T. pallidum* (Nichols) organisms were obtained through passage in rabbit testes and donated by Nigel Appleton (Newmarket Laboratories Ltd, Suffolk, UK). DNA from *T. pallidum* subspecies *pertenue* and *Treponema denticola* was donated by Sheila Lukehart (University of Washington, Seattle, USA). All other samples were donated by Stuart Philip (St Mary’s Hospital, London, UK). Human DNA samples were obtained from blood donated by patients seronegative for *T. pallidum*.

Statistical analysis was performed using PASW statistics, V18, SPSS, Chicago, IL. Significance levels were calculated using the Mann–Whitney U test (two sided, 95% CIs).

**RESULTS**
During the validation, as previously described in a rabbit model, extraction and quantification of *tpp47* DNA from WB gave more consistent results than from peripheral blood mononuclear cells or plasma (data not shown). Thus, only ulcer and WB results are presented.

Table 2 details all patients included in the study and gives serological, DGM and PCR results.

**Species specificity**
Amplification was observed neither with DNA extracted from the panel of STI and other organisms nor with human DNA. *T. pallidum* subspecies *pertenue* was detected by the assay. This result was expected, as there are very few genetic differences between *T. pallidum* subspecies.

**Sensitivity and specificity of *tpp47* detection in ulcer and WB samples**
Patients’ final clinico-microbiological diagnoses (as detailed in table 1) were compared with *T. pallidum* qPCR results.

The sensitivity and specificity for *T. pallidum* detection in syphilitic ulcers, primary or secondary, were 100% and 97-14%, respectively. The details of a potential false-positive result are given below. With the exception of secondary syphilis (57.89%), sensitivity for *T. pallidum* in WB samples was low (34.09% for all stages overall) while specificity was 100%. The sensitivity and specificity of WB PCR compared with serology for the diagnosis of primary disease were 27.27% and 100%, respectively.

All 14 patients with primary syphilis had PCR-positive ulcers and 10 were DGM positive. All four patients with DGM-negative ulcers had positive serology at enrolment. Compared with the final diagnosis, the sensitivity and specificity of DGM for syphilis ulcers (primary and secondary combined) were 70.59% and 87.88%, respectively, with no significant difference observed for primary or secondary cases analysed individually. The sensitivity of PCR compared with DGM for the diagnosis of primary disease was 100%.

**Primary syphilis**
*tpp47* DNA was detected in ulcer exudates of all 14 patients diagnosed with primary syphilis. Median copy number of *tpp47* DNA from ulcers was 1832 copies/strip (range 251–14244), which did not differ significantly between those processed immediately ((n =9) median 2577 copies/strip (427–14244)) and those processed after storage ((n=5) median 1064 copies/strip (251–2115) (p=0.162)). The median number of ulcer *tpp47* copies/strip in HIV-1-positive and HIV-1-negative patients were 2115 (757–14244) and 648 (251–2742) (p=0.048), respectively. Mean CD4 count of the seven HIV-1-infected patients was 501 (range 270–920) and CD4 count was not associated with *T. pallidum* load.

Three patients had *tpp47* DNA detectable in WB with a copy number 1–2 logs lower (median 127 copies/ml, 127–168) than for ulcers. It did not differ according to HIV status and was associated with a modal RPR of 4. The mean duration of symptoms for these patients was 8 days (range 7–10 days), compared with 30.5 days (1–90) for those who were *tpp47* DNA PCR negative in WB.

**Secondary syphilis**
*tpp47* DNA was detected in the blood of 11/19 (58%) patients with secondary syphilis. This rate of detection was higher (86%) in samples that had been processed immediately compared with 42% when DNA extraction was performed after storage (p=0.02) (see online supplementary figures). The mean *tpp47* DNA copy number in WB samples extracted immediately was 516 copies/ml and the modal RPR for these patients was 128.
Table 2 Standard clinical investigation and study results for all participants categorised by final clinico-microbiological diagnosis

| Final diagnosis               | Patient | HIV-1 status | CD4 count | DGM result* | HSV PCR result* | Syphilis EIA result at enrolment | RPR result at enrolment | Timing of DNA extraction | WB tpp47 (copies/ml whole blood) | Ulcer tpp47 copy number (copies/strip) |
|------------------------------|---------|--------------|-----------|-------------|-----------------|----------------------------------|------------------------|--------------------------|-------------------------------|--------------------------------------|
| Primary syphilis (n=14)      | 6       | –            | +         | –           | +               | 8                                | Immediate              | 0                        | 620                           |                                      |
|                             | 9       | +            | 390       | +           | 16              | Immediate          | 127                    | 2108                     | Unavailable                  |                                      |
|                             | 11      | +            | 480       | +           | 16              | Immediate          | 0                      | 12 440                    | Unavailable                  |                                      |
|                             | 18      | +            | 270       | –           | +               | 0                                | Immediate              | 0                        | 757                           |                                      |
|                             | 23      | –            | –         | +           | +               | 4                                | Immediate              | 0                        | 2742                          |                                      |
|                             | 24      | –            | –         | +           | 16              | Immediate          | 0                      | 2577                     | Unavailable                  |                                      |
|                             | 26      | +            | 920       | +           | –               | Immediate          | 0                      | 7426                     | Unavailable                  |                                      |
|                             | 32      | +            | 720       | –           | +               | 4                                | Immediate              | 168                      | 14 244                       |                                      |
|                             | 33      | –            | –         | –           | +               | 64                                | Immediate              | 0                        | 427                           |                                      |
|                             | 52      | +            | 320       | +           | +               | 2                                | Later                  | 0                        | 2115                          |                                      |
|                             | 69      | –            | –         | +           | +               | 32                                | Later                  | 0                        | 251                           |                                      |
|                             | 89      | +            | 420       | +           | –               | +                                | Later                  | 0                        | 1555                          |                                      |
|                             | 90      | –            | –         | –           | +               | 4                                | Later                  | 127                      | 1064                          |                                      |
|                             | 93      | –            | –         | +           | –               | +                                | Later                  | 0                        | 648                           |                                      |
| Secondary syphilis (n=19)    | 3       | +            | 660       | Unavailable  | +               | 64                                | Immediate              | 0                        | 2                             |                                      |
|                             | 16      | +            | 240       | –           | –               | +                                | 128                    | Immediate                | 886                           | 630                                  |
|                             | 17      | –            | –         | +           | –               | +                                | 128                    | Immediate                | 1026                          | 422                                  |
|                             | 22      | –            | –         | +           | –               | +                                | 64                     | Immediate                | 518                           | 211†                                 |
|                             | 27      | +            | 680       | +           | 8               | Immediate          | 0                      | 632                     | Unavailable                  |                                      |
|                             | 29      | –            | –         | –           | +               | 16                                | Immediate              | 58                       | Unavailable                  |                                      |
|                             | 34      | +            | 390       | Unavailable  | –               | +                                | 128                    | Immediate                | 495                           | 251†                                 |
|                             | 45      | +            | 350       | +           | 64              | Later                  | 6                      | Later                    | 0                             |                                      |
|                             | 46      | +            | 330       | +           | 16              | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 47      | –            | –         | +           | 256             | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 49      | +            | 630       | +           | 128             | Later                  | 10                     | Later                    | 0                             |                                      |
|                             | 53      | +            | 400       | +           | 64              | Later                  | 29                     | Later                    | 0                             |                                      |
|                             | 61      | +            | 470       | +           | 512             | Later                  | 91                     | Later                    | 0                             |                                      |
|                             | 63      | +            | 790       | +           | 32              | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 74      | +            | 410       | +           | 128             | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 75      | +            | 470       | +           | 256             | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 77      | +            | 370       | +           | 32              | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 81      | +            | 430       | +           | 128             | Later                  | 65                     | Later                    | 0                             |                                      |
|                             | 83      | +            | 420       | +           | 128             | Later                  | 0                      | Later                    | Unavailable                  |                                      |
| Other syphilis (latent or symptomatic late) (n=8) | 1       | –            | –         | +           | 0               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 12      | +            | 400       | +           | 1               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 28      | +            | 220       | +           | 256             | Immediate          | 70                     | Later                    | 0                             |                                      |
|                             | 44      | –            | –         | +           | 16              | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 59      | +            | 610       | +           | 0               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 67      | –            | –         | +           | 0               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 84      | +            | 570       | +           | 0               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 95      | –            | –         | +           | 1               | Later                  | 0                      | Later                    | 0                             |                                      |
| Asymptomatic contact of syphilis (n=17) | 2       | –            | –         | +           | 1               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 4       | –            | –         | +           | 0               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 8       | –            | –         | –           | –               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 15      | +            | 730       | –           | –               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 25      | –            | –         | –           | –               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 30      | +            | 320       | –           | –               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 38      | +            | 490       | +           | 0               | Immediate          | 0                      | Later                    | 0                             |                                      |
|                             | 51      | –            | –         | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 57      | –            | –         | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 58      | +            | 470       | +           | 0               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 65      | +            | 490       | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 73      | –            | –         | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 76      | +            | 670       | +           | 0               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 78      | +            | 510       | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 88      | +            | 320       | +           | 0               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 94      | +            | 640       | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |
|                             | 99      | –            | –         | –           | –               | Later                  | 0                      | Later                    | 0                             |                                      |

Continued
**Table 2 Continued**

| Final diagnosis       | Patient | HIV-1 status | CD4 count | DGM result* | HSV PCR result* | Syphilis EIA result at enrolment | RPR result at enrolment | Timing of DNA extraction | WB tpp47 (copies/ml whole blood) | Ulcer tpp47 copy number (copies/strip) |
|-----------------------|---------|--------------|-----------|-------------|----------------|----------------------------------|-------------------------|---------------------------|-----------------------------------|--------------------------------------|
| HSV infection         | 10      | –            | –         | –           | +              | –                                | –                       | Immediate                 | 0                                 | 0                                    |
|                       | 19      | –            | –         | –           | –              | +                                | –                       | Immediate                 | 0                                 | 0                                    |
|                       | 20      | –            | –         | –           | Unavailable    | +                                | –                       | Immediate                 | 0                                 | 0                                    |
|                       | 31      | –            | –         | –           | +              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 37      | –            | –         | –           | +              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 39      | –            | –         | –           | +              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 40      | –            | –         | –           | +              | +                                | 0                       | Later                     | 0                                 | 0                                    |
|                       | 41      | –            | –         | –           | +              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 42      | +            | 780       | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 43      | –            | –         | –           | +              | Unavailable                      | Later                   | 0                         | 0                                 | Unavailable                         |
|                       | 55      | +            | 550       | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 70      | –            | –         | –           | –              | +                                | –                       | Later                     | 0                                 | 0                                    |
|                       | 71      | +            | 680       | –           | –              | +                                | –                       | Later                     | 0                                 | 0                                    |
|                       | 72      | –            | –         | –           | +              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 79      | –            | –         | –           | +              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 86      | –            | –         | –           | +              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 91      | +            | 370       | Unavailable | +              | –                                | Later                   | 0                         | Unavailable                         |
|                       | 92      | –            | –         | –           | +              | +                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 98      | –            | –         | –           | +              | +                                | Later                   | 0                         | 0                                 | 0                                    |
| Non-specific genital ulceration (n=19) | 5       | –            | –         | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 7       | +            | 540       | –           | +              | +                                | 0                       | Immediate                 | 0                                 | 0                                    |
|                       | 13      | –            | –         | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 14      | –            | –         | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 21      | –            | –         | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 35      | –            | –         | –           | –              | –                                | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 48      | –            | –         | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 50      | –            | –         | –           | +              | –                                | +                       | 0                       | Later                             | 0                                    |
|                       | 54      | +            | 210       | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 60      | +            | 750       | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 62      | +            | 400       | –           | –              | –                                | Later                   | 0                         | 0                                 | 46‡                                |
|                       | 64      | +            | 130       | –           | –              | +                                | 0                       | Later                     | 0                                 | 0                                    |
|                       | 66      | +            | 590       | –           | –              | +                                | 0                       | Later                     | 0                                 | 0                                    |
|                       | 68      | +            | 580       | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 80      | –            | –         | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 82      | +            | 340       | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 85      | +            | 120       | –           | –              | +                                | 1                       | Later                     | 0                                 | 0                                    |
|                       | 87      | +            | 610       | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
|                       | 97      | +            | 500       | –           | –              | –                                | Later                   | 0                         | 0                                 | 0                                    |
| Neurosyphilis (n=3)   | 36      | +            | 290       | –           | +              | 32                               | Immediate               | 0                         | 0                                 | 0                                    |
|                       | 56      | –            | –         | +           | 64              | –                                | Late                    | 0                         | 0                                 | 0                                    |
|                       | 96      | +            | 660       | +           | 8               | –                                | Late                    | 0                         | 0                                 | 0                                    |

*Test performed only when clinically indicated.
‡Muco-cutaneous oral lesion.
§Patient with a 1-week history of multiple non-tender genital ulcers thought to be atypical genital herpes and treated with acyclovir. Convalescent syphilis serology and PCR for HSV types 1 and 2 were negative.

DGM, dark ground microscopy; EIA, enzyme immunoassay; HSV, herpes simplex virus; RPR, rapid plasma reagin; WB, whole blood.

T pallidum was detected in WB of 8/15 (53%) HIV-1-infected versus 3/4 (75%) uninfected (28%) patients. Mean CD4 count of HIV-1-infected patients with tpp47-positive WB was 448, not significantly different from those tpp47 negative in WB (492, p=0.594).

Three patients had muco-cutaneous oral lesions, of which two were positive for tpp47 DNA. Four patients with secondary syphilis still had anogenital ulcers of which three were sampled and all were tpp47 DNA positive. When compared, the mean tpp47 DNA copy number was lower in these anogenital ulcers persistent in secondary disease (351 copies/strip) than in primary chancres (3498 copies/strip) (p=0.052).

**Other cases**

tpp47 DNA was detected in WB of one of the eight patients with latent disease. This HIV-1-positive untreated patient, with an initial RPR titre of 1:131 072, had been lost to follow-up for 2 years prior to the study. RPR at the time of recruitment was 1:256.

One ulcer sample from a patient with a 1-week history of multiple non-tender genital ulcers thought to be atypical genital herpes and treated with acyclovir had detectable HSV DNA. tpp47 DNA was negative as were the contemporaneous and convalescent syphilis serology and PCR for herpes simplex virus types one and two. This may represent a false-positive PCR result, but it is not known whether this patient received...
coincidental treponemocidal antibiotics that could have prevented seroconversion.

CONCLUSIONS AND DISCUSSION

For 20 years, PCR has been used with a variety of clinical and rabbit specimens to detect, type and quantify T pallidum.\(^5\)\(^6\)\(^10\)\(^20\)\(^21\) PCR reliably detects T pallidum in ulcer specimens and is now established, often as part of a multiplex assay, for the diagnosis of primary syphilis.\(^5\) The more modest success of T pallidum DNA PCR in other clinical samples has been attributed to their lower treponemal load.\(^9\)

There are few data on the quantification of T pallidum by PCR in clinical samples, thus the timing of bacteraemia and duration of post-treatment as well as the impact of HIV-1 infection on the course of T pallidum infection are uncertain. Although cross-sectional, the data presented here provide insight into the range of T pallidum DNA copies that can be detected in samples taken at different stages of disease.

Since several groups had found the tpp47 gene to be reliable and specific for T pallidum detection,\(^5\)\(^9\)\(^12\)\(^13\) new primers were designed for a real-time PCR assay. These have been shown to be equally specific for T pallidum detection with no cross-reactivity to T denticola, any of the common commensal skin flora or sexually transmitted organisms tested. Sensitivity was maintained when used to detect T pallidum DNA extracted from human WB samples with no inhibition by the high concentration of human DNA.

Storage at \(-20^\circ C\) prior to DNA extraction impaired the detection and quantification of T pallidum in WB but not in ulcer samples. Cruz \textit{et al} reported a similar effect, with detection of T pallidum DNA in WB of patients with secondary syphilis just 30% compared with 63% when the samples were processed within a few hours of collection.\(^23\) The maximum interval between blood sampling and DNA extraction, which is likely to be short, remains to be established. Additional study is also needed to optimise T pallidum collection from ulcer samples. While Sno Strips are shown here to be efficient at absorbing exudate and bacterial DNA, the volume (which is part dependent on the ulcer area) of ulcer exudate will influence final copy number in the same way as the concentration of T pallidum in the ulcer fluid. Quantifying a human reference gene from ulcers (eg, \(\beta\)-globin) and comparing it with tpp47 load may also help standardisation. It remains to be shown, however, that amount of human DNA detected would not simply mirror that of T pallidum.

T pallidum DNA was detected in the ulcers of all 14 patients diagnosed with primary syphilis, a similar finding to the work of Palmer \textit{et al} in 2002\(^2\)\(^5\) and unsurprising, given that the chance is the site of bacterial inoculation. Moreover, tpp47 DNA PCR from ulcer exudates appears to more reliably include or exclude T pallidum infection than DGM. T pallidum DNA was also detected in ulcer samples in secondary syphilis with three out of four genital and two out of three oral lesions being positive. Fewer organisms/strip were detected in these lesions than in primary chancres (351 vs 3498), which likely represents ulcer healing and resolution.

T pallidum bacteraemia is thought to occur from the outset of the primary infection and to persist at all stages of disease thereafter,\(^25\) and while these stages are well defined, the rate of bacterial dissemination in humans is unknown. In rabbits inoculated intratesticularly, T pallidum flaA DNA could be detected at low load in blood after 24 h and at much higher load after 7–10 days. The increased detection after 1 week corresponded with rising RPR titres and widespread treponemal dissemination into organs.\(^10\) The detection rates observed in WB for patients with primary (3/14, 21.4%) and secondary (11/19, 58%) syphilis are lower than the detection rates for ulcers, but are comparable with those reported previously.\(^12\)\(^22\) A higher detection rate in WB in secondary syphilis corresponds to a greater number of organisms and higher RPRs, similar to the picture reported in the rabbits 7–10 days post-inoculation. T pallidum DNA detection in blood has been reported in 0–58.3% of patients with latent disease.\(^9\)\(^14\)\(^22\)–\(^25\) Kouznetsov \textit{et al} reported higher detection of T pallidum DNA in peripheral blood mononuclear cell samples in early latent (4/4) than in late latent (1/7) cases, and in our study, quantifiable low-load (70 copies) bacteraemia was found in only one patient (1/7, 14.3%), who had early latent disease.\(^14\)

Combining the qualitative and quantitative findings, a pattern of bacterial dissemination emerges. In primary disease, high numbers of organisms in ulcers but low copy number or no tpp47 DNA were detected in blood. In secondary disease, the healing ulcers have decreased load but bacteraemia is more frequently detected and at higher load than primary disease. By the latent stage, treponemal bacteraemia is less commonly detected and when present is at low load.

Syphilis and HIV-1 are known to interact in a number of ways. Both HIV-1 transmission and acquisition are facilitated by the presence of syphilitic ulcers and there is evidence that those ulcers and the clinical course of syphilis can be affected by HIV-induced immunosuppression.\(^5\) Rompalo \textit{et al} studied 214 patients with genital ulcers and found HIV-1-positive men to be 11% more likely to have secondary syphilis with a concomitant primary chancre.\(^26\) Similarly, in this study, HIV-1-positive patients with secondary syphilis were more likely to present with unhealed primary ulcers. HIV-1-positive patients had a higher number of organisms in primary ulcers than patients known to be HIV-1 uninfected. Although this association did not reach a high level of statistical significance, the presence of more organisms suggests that HIV-1-positive patients with primary syphilis are more infectious and for longer, which may help explain the high rates of syphilis–HIV co-infection.

This study had small sample sizes in some groups, which limited the statistical significance of associations. It is also appreciated that longitudinal sampling of the same patients during the course of their infection would more accurately reflect infection kinetics but would not be ethical. Importantly though, we demonstrate that quantification of T pallidum has the potential to determine the kinetics of the infection and the impact of HIV-1 co-infection on those kinetics. In this study, a pattern of T pallidum load by site and stage is described with important observations related to the infectiousness of HIV-positive patients with primary syphilis.

**Key messages**

- Real-time PCR can be used to quantify Treponema pallidum in clinical samples and provide insight into the kinetics of syphilis infection.
- The amounts of T pallidum found in ulcers and blood at different stages of syphilis follow a biologically plausible pattern.
- Larger numbers of T pallidum in ulcers may mean HIV-1-positive patients can more easily transmit syphilis.
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Competing interests The corresponding author has had full access to all the data in the study and had final responsibility for the decision to submit for publication. These data were presented, in part, at the IUSTI World Congress, Cape Town, 2009.

Ethics approval This study was conducted with the approval of the Hounslow and Hillingdon Local Research Ethics Committee. Ref: 06/Q0407/29.

Contributors CT: Sample analysis, data collection and interpretation, and wrote the manuscript. MOFH: Developed and validated the tp47 qPCR, performed sample analysis and co-wrote the manuscript. DG: Initial study design and co-wrote the manuscript. SH and JD: Recruited the patients and collated clinical data. MOMC: Designed and performed the study and co-wrote the manuscript. GFT: Designed and supervised the study, obtained regulatory approval and co-wrote the manuscript.

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REFERENCES
1. Collart P, Borel LJ, Durel P. Significance of spiral organisms found, after treatment, in late human experimental syphilis. Br J Vener Dis 1964;40:81–9.
2. Larsen SA, Steiner BM, Rudolph AH. Laboratory diagnosis and interpretation of tests for syphilis. Clin Microbiol Rev 1995;8:1–21.
3. Zetola NM, Klauser JD. Syphilis and HIV infection: an update. Clin Infect Dis 2007;44:1222–8.
4. Handfield HH, Kuller L, Deeks SL, et al. Syphilis associated with concurrent simian immunodeficiency virus infection. J Infect Dis 1992;165:1020–5.
5. Orle KA, Larsen SA, Zetola NM, et al. Simultaneous PCR detection of Haemophilus ducreyi, Treponema pallidum, and herpes simplex virus types 1 and 2 from genital ulcers. J Clin Microbiol 1998;36:40–54.
6. Heyman R, van der Helm JJ, de Vries HJ, et al. Clinical value of Treponema pallidum real-time PCR for diagnosis of syphilis. J Clin Microbiol 2010;48:497–502.
7. Leslie DE, Azzato F, Karapanagiotidis T, et al. 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. J Clin Microbiol 2007;45:93–6.
8. Koek AG, Brustein SM, Dierdorf M, et al. Specific and sensitive diagnosis of syphilis using a real-time PCR for Treponema pallidum. Clin Microbiol Infect 2008;12:1233–6.
9. Castro R, Prieto E, Aguas MJ, et al. Detection of Treponema pallidum sp pallidum DNA in latent syphilis. Int J STD AIDS 2007;18:842–5.
10. Salazar JC, Rathi A, Michael NL, et al. Assessment of the kinetics of Treponema pallidum dissemination into blood and tissues in experimental syphilis by real-time quantitative PCR. Infect Immun 2007;75:2954–8.
11. Hare PE, Clarke JP, Stringer SA, et al. Use of the polymerase chain reaction to detect DNA sequences specific to pathogenic treponemes in cerebrospinal fluid. FEMS Microbiol Lett 1990;56:233–8.
12. Palmer HM, Higgins SP, Harrington AJ, et al. Use of PCR in the diagnosis of early syphilis in the United Kingdom. Sex Transm Infect 2003;79:479–83.
13. Burstain JM, Gremel P, Lukehart SA, et al. Sensitive detection of Treponema pallidum by using the polymerase chain reaction. J Clin Microbiol 1991;29:62–9.
14. Kouznetsov AV, Weissenseel P, Trommler P, et al. Detection 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.
15. Ross J, Ison C, Carder C, et al. Sexually Transmitted Infections: UK National Screening and Testing Guidelines, 2006. http://www.bashh.org/documents/59/59.pdf (accessed 25 Jan 2010).
16. Kingston M, French H, Goh B, et al. UK national guidelines on the management of syphilis 2008. Int J STD AIDS 2008;19:729–40.
17. Risbud A, Chan-Tack K, Gadkari D, et al. The etiology of genital ulcer disease by multiplex polymerase chain reaction and relationship to HIV infection among patients attending sexually transmitted disease clinics in Pune, India. Sex Transm Infect 1999;75:55–62.
18. O’Farrell N, Hosen AA, Coetzee KD, et al. Genital ulcer disease in men in Durban, South Africa. Genitourin Med 1991;67:327–30.
19. Health Protection Agency. Syphilis and Lymphogranuloma Venereum: Resurgent Sexually Transmitted Infections in the UK, 2009. http://www.hpa.org.uk/webc/ HPResFile/HPRes/FDF/2/C1245591513523 (accessed 25 Jan 2011).
20. Centurion-Lara A, Castro C, Shaffer JM, et al. Detection of Treponema pallidum by a sensitive reverse transcribe PCR. J Clin Microbiol 1997;35:1348–52.
21. Pillay A, Liu H, Brahman S, et al. Molecular typing of Treponema pallidum in South Africa: cross-sectional studies. J Clin Microbiol 2002;40:256–8.
22. Cruz AR, Pillay A, Zhuang M, et al. Secondary syphilis in Cali, Colombia: new concepts in disease pathogenesis. PLoS Negl Trop Dis 2010;4:e690.
23. Marin AA, Liu H, Sutton MY, et al. Amplification of the DNA polymerase I gene of Treponema pallidum from whole blood of persons with syphilis. Diagn Microbiol Infect Dis 2001;40:163–6.
24. 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.
25. Orton SL, Liu H, Dodd RY, et al. Prevalence of circulating Treponema pallidum DNA and RNA in blood donors with confirmed-positive syphilis tests. Transfusion 2002;42:94–9.
26. Rompalo AM, Lawlor J, Seaman P, et al. Modification of syphilitic genital ulcer manifestations by coexistent HIV infection. Sex Transm Dis 2001;28:448–54.

Book review

Sexually Transmitted Infections: The Facts (3rd edition)
Edited by David Barlow (with Dr Julie Fox, for chapter on HIV and AIDS—the clinical picture). Published by Oxford University Press, Oxford, 2011, pp 152. £12.99 (http:// ukcatalogue.oup.com/product/9780199565535.do (softback). ISBN 978-0-19-956556-5).

This little book provides detailed information on sexually transmitted infections (STIs) and related conditions, but does not stop there. It begins by explaining what patients can expect when they go for STI testing, what a genitourinary medicine clinic is and why they might choose to attend one. A chapter entitled ‘Understanding your results’ explains complex concepts, including false positives and negatives and the implications of test sensitivity and specificity. It also covers issues in the interpretation of surveillance data (such as how changes in testing patterns and practices can lead to apparently higher rates of infection). Chapters on different infections follow, and the book concludes with two chapters on HIV/AIDS, one putting the HIV epidemic in its social and historical context, and the other offering a clinical overview of HIV testing, infectivity, postexposure prophylaxis and treatment. Although adults and young people may increasingly seek out information on STIs online, there is certainly still a place for the third edition of this book, which is clearly written and authoritative. I would hope to see this book in college, secondary school or public libraries; despite the depth and detail provided, it is still small enough to hide under homework or revision notes! It would be a useful resource for both staff and young people in youth services and students’ unions. Academic researchers new to the field of STIs may also benefit from the overview the book provides on specific infections, testing practices and how genitourinary services operate—which may seem obvious to their genitourinary clinical colleagues. At the end of each chapter, a few references are supplied; for instance, URLs for BASHH/IUSTI clinical guidelines and patient organisations such as the Herpes Virus Association and Terrence Higgins Trust. The tone is matter-of-fact, if slightly dated in places. With considerable effort taken to convey a large amount of information in such a short volume, one can forgive the occasional lapse into technical terminology (in most cases this is explained). Light-hearted cartoons make a charming and welcome change from the graphic photos that one might expect in a book on STIs, which I felt to be appropriate, since photos may (in my view) reinforce public misconceptions that serious infections are obvious and symptomatic. Finally, historical anecdotes and patient vignettes provide human interest in this useful, educational book.

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