Original research

# Timing of primary syphilis treatment and impact on the development of treponemal antibodies: a cross-sectional clinic-based study

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## ABSTRACT

**Background** Serology is negative in a proportion of primary syphilis cases where *Treponema pallidum* PCR testing is positive. We aimed to identify discordant, *T. pallidum* PCR-positive, serology-negative primary syphilis cases and any clinical or laboratory factors associated with failure to subsequently seroconvert.

**Methods** Serodiscordant primary syphilis cases that were *T. pallidum* PCR-positive and serology-negative (including rapid plasma reagin, *T. pallidum* particle agglutination, *T. pallidum* enzyme immunoassay or *T. pallidum* chemiluminescence assay) were identified from the Melbourne Sexual Health Centre electronic records between April 2011 and December 2019. Clinical and laboratory associations were examined.

**Results** There were 814 primary syphilis cases in the study period and 38 (4.7%) were serodiscordant, 35 in men who have sex with men. Thirty-two had follow-up serology performed a median of 24 days later, of which 16 (50%) seroconverted, mostly (81%) within 6 weeks. Failure to seroconvert was significantly associated with treatment on day 1. Of the 12 cases treated on day 1, 10 (83%) failed to seroconvert compared with 6 of 20 (30%) among those who were treated after day 1.

**Discussion** Earlier treatment of primary syphilis can prevent the development of serological markers. *T. pallidum* PCR can identify primary syphilis lesions before the development of serological markers and improve diagnosis of early primary syphilis lesions. Serology alone will miss a proportion of primary syphilis infections and should be repeated if a diagnosis of syphilis is being considered.

## INTRODUCTION

Syphilis cases continue to rise globally, including increases among men who have sex with men (MSM) as well as heterosexuals in high-income countries.[1–3] Serological testing for syphilis is negative in a proportion of primary syphilis cases, and these diagnoses will be missed if serology alone is used in the diagnosis of anogenital lesions.

Serology has been the mainstay of syphilis screening and diagnosis since the development of the Wassermann test almost 120 years ago.[4 5] PCR assays for the direct detection of *Treponema pallidum* DNA have been available since the 2000s and have higher sensitivity in detection of *T. pallidum* DNA in primary syphilis lesions than dark-field microscopy (DFM).[6 7] *T. pallidum* DNA detection by PCR can be particularly useful in early primary syphilis before a serological response has developed,[8] and in syphilis reinfection where specific syphilis treponemal tests may remain positive for life.[5] Some studies have shown rapid plasma reagin (RPR) tests to be negative in up to 20% of primary syphilis cases where the infection has been confirmed by *T. pallidum* PCR.[9–11] Less frequently, *T. pallidum* PCR results may be positive in early syphilis before the development of both non-treponemal and treponemal results.[8 12] Since the introduction of a *T. pallidum* PCR assay in 2003,[13] we identified a number of discordant cases with *T. pallidum* PCR-positive lesions and negative serological testing for syphilis. To further investigate these, a retrospective study was performed with the aim of identifying any seronegative primary syphilis infections and any possible clinical or laboratory factors associated with failure to subsequently seroconvert.

## METHODS

### Study setting

This study was conducted at the Melbourne Sexual Health Centre (MSHC), the major public STI clinic in Victoria, Australia. Patients attending the clinic with anogenital lesions had swabs routinely collected from genital or anal lesions, such as ulcers, by a sexual health clinician for *T. pallidum* PCR and herpes simplex virus PCR, placed in transport medium and sent at room temperature on the same day to the Victorian Infectious Diseases Reference Laboratory (VIDRL). In addition, patients presenting with anogenital lesions routinely had serological testing for syphilis on the same day.

### Case definition

All MSHC primary syphilis cases diagnosed between April 2011 and December 2019 were identified from the MSHC medical records. At MSHC the syphilis stage was recorded in an electronic database by a sexual health physician after PCR and serology results were known. We identified and

extracted all the cases with a discordant (positive *T. pallidum* PCR result and negative syphilis serology) collected on the same date from the MSHC electronic results database. The date of swab collection was denoted as day 1. Any subsequent syphilis serology from these cases was also identified.

In Australia, the Department of Health Public Health Laboratory Network (PHLN) syphilis case classification has 'definitive' and 'suggestive' criteria for the laboratory diagnosis of acquired active syphilis.[14] The definitive PHLN criteria for laboratory diagnosis of active acquired syphilis are reactive non-treponemal test such as RPR or Venereal Disease Research Laboratory (VDRL), plus at least one reactive specific treponemal test such as *T. pallidum* particle agglutination assay (TPPA), chemiluminescence immunoassay (CLIA) or enzyme immunoassay (EIA), *or* demonstrated seroconversion of a specific treponemal test within 12 months of a negative test, *or* detection of *T. pallidum* by nucleic acid test from a suitable clinical specimen including cerebrospinal fluid (CSF), tissue and chancre.[14] The suggestive PHLN criteria for laboratory diagnosis of active acquired syphilis include DFM of lesion exudate showing the characteristic morphology and motility of *T. pallidum*. The US Centers for Disease Control and Prevention (CDC) also describes confirmatory and supportive laboratory criteria for the diagnosis of primary syphilis.[15] The CDC confirmatory criteria are demonstration of *T. pallidum* by DFM in a clinical specimen that is not obtained from the oropharynx and is not potentially contaminated by stool, *or* demonstration of *T. pallidum* by PCR or equivalent direct molecular methods in any clinical specimen.[15] The CDC supportive criteria are reactive non-treponemal serological test (RPR or VDRL), *or* reactive treponemal serological test including TPPA, chemiluminescence imunoassays (CMIA) or EIA.[15] All cases in this report were consistent with both the definitive diagnostic criteria of the PHLN syphilis laboratory case definition for acquired active syphilis as well as the CDC confirmatory laboratory criteria for primary syphilis.[14 15]

Shields *et al*[8] used a subsequent 6-week cut-off period for seroconversion to occur as being diagnostic of syphilis infection at the date of the *T. pallidum* positive PCR result. This 6-week period was based on the minimum time for someone to be treated for syphilis, 'wash out' time of doxycycline or penicillin, time to be reinfected with syphilis and time to then mount a new serological response.[8] We have used the same 6-week seroconversion cut-off to determine whether seroconversion occurred or not. For each patient with a discordant result, two sexual health physicians (JMT and ID) retrospectively reviewed the medical records and made an assessment as to whether the positive *T. pallidum* result likely represented a confirmed or possible primary syphilis infection. A confirmed primary syphilis case in this study was defined as a positive *T. pallidum polA* PCR result and at least one other of the following positive results: a second positive *T. pallidum* PCR assay using a different (*47 kDa*) gene target, *or T. pallidum* seen on DFM, *or* reactive serological testing for syphilis using one or more of the serological assays within 6 weeks of the day of swab collection, that is, day 1. A possible primary syphilis case in this study was defined as an isolated *T. pallidum polA* PCR result and/or seroconversion greater than 6 weeks after swab collection on day 1.

### Dark-field microscopy

Clinicians collected fresh lesion exudate from suitable anogenital ulcers onto a glass slide with a cover slip for *T. pallidum* DFM examination. At MSHC, staff in an onsite laboratory of the Microbiological Diagnostic Unit (MDU), University of Melbourne performed the DFM examinations immediately following specimen collection. Staff from MDU are skilled and experienced in the performance of DFM and in differentiating the motility and morphology of *T. pallidum* from commensal spirochaete species.

### PCR testing

Total DNA was extracted from fresh specimens on the Freedom EVO 100 (Tecan, Mannedorf, Switzerland) using the NucleoMag 96 Virus Extraction Kit (Macherey-Nagel, Duren, Germany), as per the manufacturer's instructions. DNA extracts were stored at −20°C, and the original lesion and non-lesion study samples stored at −70°C.

All samples were tested with a TaqMan real-time PCR assay targeting the *T. pallidum polA* gene with a threshold cycle ($C_T$) cut-off of 38.[13] The following modifications were made to the protocol described by Leslie *et al*.[13] Real-time PCR mixtures contained 0.3 µM concentrations of each primer, 0.15 µM concentration of the probe, 1X Premix Ex Taq (Takara, Kusatsu, Japan), and the internal DNA positive control (Bioline, London, UK) in a total reaction volume of 20 µL. Specimens were tested in duplicate with a $C_T$ cut-off of 38. Samples that produced a $C_T$ cut-off of 39–40 were retested, and if the same result was generated the sample was reported as a positive sample.

The $C_T$ value is the number of PCR cycles required to detect *T. pallidum* in the specimen, with lower values reflecting larger target DNA quantities and higher values reflecting lower amounts of target DNA.

Most samples were also tested with a second *T. pallidum* PCR assay targeting the *47 kDa* major lipoprotein immunogen of *T. pallidum*.[16] The *47 kDa* assay was designed using the Primer Express software program (Applied Biosystems, Foster City, California) targeting a 61 bp sequence (nucleotides 894–955; GenBank accession number M88769.1) within the *47 kDa* antigen gene of *T. pallidum*. Primers were synthesised by Bioneer (Bioneer, Daejeon, Korea) and the MGB TaqMan Probe was synthesised by Applied Biosystems. The probe was labelled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5' end and a non-fluorescent quencher at the 3' end. The following are the sequences for primers and probe: forward primer Syph *47 kDa* TF 5'-TGACGCGAGCTACACCAATC-3', reverse primer Syph *47 kDa* TR 5'-ACCAGGAGTCAGCAGAGTGCTT3' and MGB Probe Syph *47 kDa* TP 5'-6FAM-ATGGCGAGTTATGGCAC-MGB-NFQ. The PCR mixture contained template DNA and identical primer, probe, premix and internal DNA positive controls to the *polA* assay. Amplification and detection were performed on the CFX96 Real-Time Detection System (BioRad, California, USA), using 1 cycle at 95°C for 3 min followed by 40 cycles at 95°C for 3 s and 60°C for 30 s.

### Syphilis serology

Sera were tested with RPR (Becton Dickinson, New Jersey, USA) and TPPA assays (Fujirebio, Tokyo, Japan). Prior to January 2016 sera were tested with a recombinant total antibody ELISA immunoassay (EIA) (Trepanostika EIA, BioMerieux, Marcy-l'Etoile, France). From January 2016, sera were tested with LIAISON Treponema Screen (DiaSorin, Saluggia, Italy), an automated CLIA. Selected sera were also tested with a *T. pallidum* IgM assay. Prior to 15 October 2014, the BioRad Syphilis IgM EIA was in use. This assay was then replaced with the Euroimmun Anti-*Treponema pallidum* ELISA (IgM), which was used for the remainder of the study. VIDRL used an algorithm based on reverse screening, where the EIA or CLIA assay is performed

first and then other serological tests performed if the EIA/CLIA is positive or if there is a positive *T. pallidum* PCR from the same case. At times there is variation from this general approach depending on clinical information provided by the requesting clinician. In every case, the baseline EIA (total) or CLIA, TPPA and RPR results (on day 1) were from testing of the same sera, which were collected on day 1, the same day of sampling the *T. pallidum* PCR-positive lesions.

### Statistical analysis

Data were analysed using SPSS V.25. Categorical variables, such as seroconversion status and day of treatment, were compared using Fisher's exact test, while continuous variables, such as time to treatment and duration of symptoms, were compared using non-parametric Mann-Whitney U test.

## RESULTS

### Clinical characteristics

Between April 2011 and December 2019, there were 814 primary syphilis cases diagnosed at MSHC. Of these, 38 (4.7%; 95% CI 3% to 6%) cases were discordant on the day of the first presentation, that is, negative on syphilis serology and positive by *T. pallidum* PCR. Thirty-seven discordant cases were male and one was female. Of the male cases, 35 (95%) were MSM and 2 (5%) were men who have sex with women only (figure 1). Three were HIV-positive, all MSM and on HIV antiretroviral treatment, with CD4 counts >500/mm$^3$ and HIV viral load of <50 copies/mL. The sites of the sampled lesions were 30 penile, 7 perianal and 1 vulval. On the day of first attendance, all 38 cases had RPR, 25 had CLIA total antibody or 13 EIA total antibody performed, and 37 cases also had TPPA performed. The duration of the lesions was reported for 36, with a median duration of 3 days (IQR 2–7 days). There was no significant difference between the duration of symptoms at different sites (p=0.25). Of the 38 cases, 32 had syphilis serology repeated at the second visit. Six cases which did not return for any follow-up are discussed but were excluded from the analyses. Detailed clinical and laboratory characteristics of all 38 cases are shown in online supplemental table 1.

### Cases that seroconverted

Of the 32 cases with follow-up serology, 16 (50%) had seroconverted when serology was subsequently repeated: 12 within 6 weeks, and 4 by days 57, 87, 212 and 229 days. Of the 16 cases that seroconverted, the following serological markers were positive: TPPA (16 of 16), EIA (2 of 4) or CLIA (8 of 12), EIA IgM (6 of 13), and RPR (6 of 16). RPR values ranged between 1:1 and 1:8. An additional three cases had equivocal CLIA results. The cases that seroconverted but that had a negative EIA or CLIA were all collected at or less than 9 days after day 1.

Of the four with delayed seroconversion, three had a positive *47 kDa* result (one of which was positive by DFM) and the fourth did not have either *47 kDa* assay or DFM performed. One had an RPR value of 1:1 and three had a non-reactive RPR.

### Cases with no seroconversion

Of the 32 cases with follow-up serology, there were 16 cases where seroconversion did not occur. Ten were treated on day 1 and had either a chancre, positive DFM or were a reported contact of syphilis. The remaining six were treated between days 8 and 18. The 16 cases with no seroconversion had the following tests performed at follow-up: EIA (8 of 16) or CLIA (8 of 16), TPPA (12 of 16), and RPR (14 of 16). Of the 16 cases with no seroconversion, 12 were confirmed with a positive *T. pallidum 47 kDa* and/or positive DFM. Four cases were deemed to be possible primary syphilis and treated as such. They all had positive *T. pallidum polA* PCR results but no other confirmatory results.

Failure to seroconvert was more likely to occur if syphilis treatment occurred on day 1 compared with treatment after day 1. If treated on day 1, 10 of 12 (83%) did not seroconvert compared with those treated after day 1, where 6 of 20 (30%) did not seroconvert (p=0.009).

The clinical and laboratory characteristics of *T. pallidum* PCR-positive, seronegative lesions among cases by subsequent seroconversion status are shown in table 1.

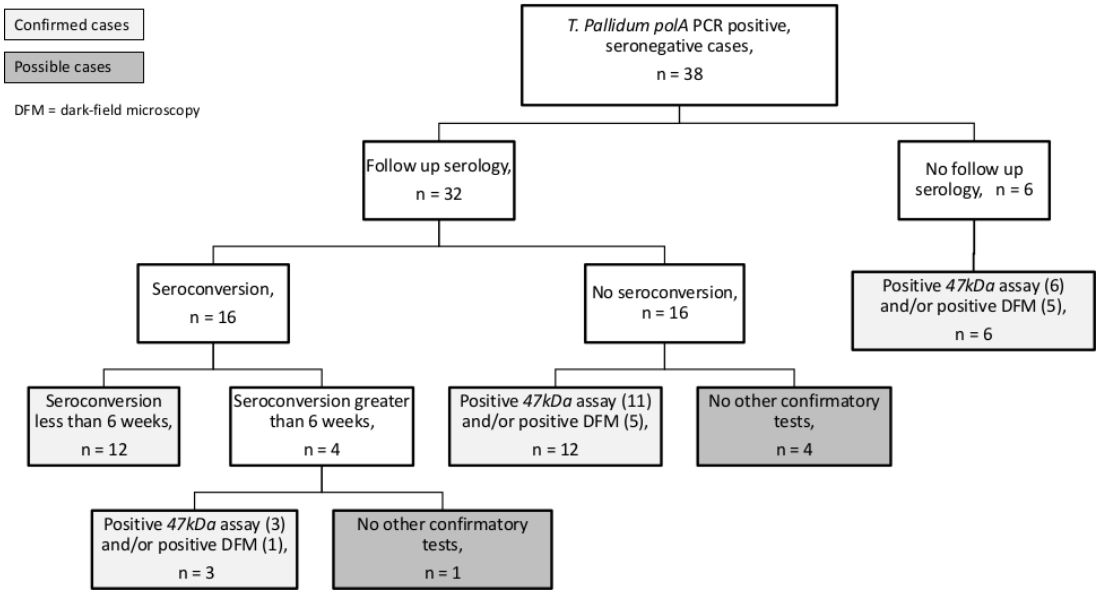


**Figure 1** *Treponema pallidum polA* PCR-positive and syphilis serology-negative cases.

**Table 1** Clinical and laboratory characteristics of *Treponema pallidum* PCR-positive, seronegative lesions among cases by subsequent seroconversion status

| | Seroconversion (n=16) | No seroconversion (n=16) | Total (n=32) | |
|---|---|---|---|---|
| | n (%) | n (%) | n (%) | P value |
| MSM | 15 (94) | 14 (88) | 29 (91) | 1 |
| Known contact of syphilis | 1 (6) | 3 (19) | 4 (12.5) | 0.60 |
| HIV-1-positive | 1 (6) | 2 (13) | 3 (9.4) | 1 |
| Penile lesion | 14 (88) | 10 (63) | 24 (75) | 0.22 |
| Perianal lesion | 2 (13) | 5 (31) | 7 (22) | 0.39 |
| Painful lesion | 7 (44) | 7 (44) | 14 (44) | 1 |
| Multiple lesions | 8 (50) | 8 (50) | 16 (50) | 1 |
| Treatment on day 1 | 2 (12.5) | 10 (62.5) | 12 (37.5) | 0.009 |
| Symptom duration, days, median (range)* | 4 (1–14) | 3 (1–7) | 4 (1–14) | 0.63 |
| Treatment day, median (range) | 8 (1–20) | 1 (1–18) | 7.5 (1–20) | 0.09 |
| DFM-positive† | 1 (13) | 5 (71) | 6 (40) | 0.04 |
| Herpes simplex virus-positive | 1 (6) | 3 (19) | 4 (12.5) | 0.60 |
| *T. pallidum polA* $C_T$ value, median (range) | 35.5 (25–38) | 34 (27–37) | 34 (25–38) | 0.18 |
| *T. pallidum 47 kDa* assay-positive‡ | 13 (100) | 11 (79) | 24 (89) | 0.22 |
| *T. pallidum 47 kDa* $C_T$ value, median (range) | 36 (28–40) | 35 (30–39) | 35 (28–40) | 0.90 |
| Follow-up serology day, median (range) | 10.5 (6–228) | 41 (7–307) | 23.5 (6–307) | 0.02 |

*Two cases did not have symptom duration recorded.
†Seventeen cases did not have DFM performed.
‡Five cases did not have *T. pallidum 47 kDa* PCR performed.
$C_T$, cycle threshold; DFM, dark-field microscopy; MSM, men who have sex with men.

### Cases with no follow-up serology

The six cases with no follow-up serology all received syphilis treatment on day 1. All six cases were positive on both the *T. pallidum polA* and *47 kDA* assays. Five were also positive on DFM.

## DISCUSSION

In our study, we showed that among primary syphilis infections that were *T. pallidum* PCR-positive and negative by serological testing, timing of treatment was significantly associated with subsequent seroconversion, with cases receiving syphilis treatment on the day of first presentation being more likely to remain seronegative. The majority of the initially seronegative cases (33 of 38, 87%) were confirmed as syphilis infection by either seroconversion within the subsequent 6 weeks, DFM or by a second positive *T. pallidum* PCR with a different gene target.

A strength of this study is that we used two different *T. pallidum* PCR assays, each with a different gene target, to increase the likelihood of identifying a true positive result and identify those cases which may have been due to a false positive *T. pallidum polA* assay result. A previous study found no difference in the sensitivity of these two PCR assays.[17] A limitation of this study was that it was a retrospective analysis and the timing of follow-up serology was variable and not all assays were performed in follow-up of all cases. In addition, some of the follow-up periods were short, and if longer it is possible that the rates of seroconversion would have been higher.

There are a number of other possible factors that might contribute to a failure to seroconvert, including exposure to other antibiotics around the time of initial presentation, low lesion load of *T. pallidum* which may have delayed or not triggered a serological response, and use of less sensitive serological assays. In addition, results may vary in different settings; for example, in settings where primary infection is more likely to present later in the course of disease, rates of seronegative would likely be lower.

Five cases in our study were categorised as possible syphilis cases as they were unable to be confirmed by other diagnostic criteria other than a positive *T. pallidum polA* assay result. One had seroconverted by day 288 with positive TPPA, EIA and negative RPR, but a subsequent syphilis reinfection cannot be excluded. The other four may represent false positive *T. pallidum polA* PCR results. Another limitation was that DFM was only performed on a subset of the anogenital lesions. False positive DFM can result from difficulty differentiating the motility and morphology of *T. pallidum* from commensal spirochaetes of the gastrointestinal tract. However, of the 11 cases that had positive DFM results, 10 were from penile lesions and only 1 from an anal lesion.

Seronegative primary syphilis has been previously described. Two papers have reported cases of PCR-positive, serology-negative cases using specific and non-specific treponemal markers. Shields *et al*[8] described five serodiscordant primary syphilis cases and observed delayed seroconversion in four of these, with one remaining seronegative over a year later. Palmer *et al*[12] described a discordant seronegative case and hypothesised that concurrent antibiotic treatment had prevented a serological immune response. We believe our series is the largest of serodiscordant primary syphilis cases to be described and the only to show a statistical association between earlier treatment of primary syphilis and absence of seroconversion.

*T. pallidum* PCR may be positive before the development of any serological markers of syphilis, and if discordant should prompt repeat syphilis serology, particularly when atypical lesions or risk factors are present. Only the first syphilis infections were able to be identified in this way, as treponemal serology usually remains positive after the first infection, and RPR may be negative in primary syphilis as this marker may be slower to develop than specific treponemal tests such as TPPA, EIA and CLIA/CMIA.[9–11]

With syphilis being increasingly seen in many countries, it is important that sensitive nucleic acid tests for *T. pallidum* be more widely available in primary healthcare settings and sexual

health clinics, as serology alone will result in a proportion of diagnoses being missed or treatment being delayed, and therefore increasing the risk of complications and further transmission. In settings where *T. pallidum* PCR testing is not available, clinicians should consider repeating serology when syphilis is clinically suspected or there is an epidemiological risk. Training and awareness of primary healthcare providers are also important so that syphilis as a differential diagnosis is considered when patients present with an anogenital lesion and testing for *T. pallidum* using nucleic acid tests is performed. Prevention efforts should be targeted at the detection and treatment of syphilis before progression to the secondary stage, which is associated with transmission of infection between partners.[18]

## Key messages

- Primary syphilis may be diagnosed by *Treponema pallidum* PCR before the evolution of any serological markers.
- Half of the seronegative syphilis cases in this study subsequently seroconverted, most within 6 weeks.
- Treatment on day 1 was more likely to prevent seroconversion than treatment on a later date.

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**Contributors** DEL conceived and designed the study and drafted the original manuscript. FA performed the laboratory testing. Data analysis was performed by JMT. JMT and MYC drafted the final version of the manuscript. EPFC and LZ provided advice on statistical analysis. All authors except DEL (deceased in 2018) critically reviewed the manuscript for important intellectual content and approved the final version.

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