Use of PCR in the diagnosis of early syphilis in the United Kingdom

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After falling to an all time low in the early 1990s, the prevalence of infectious syphilis (primary and secondary stages) in the United Kingdom rose substantially after 1996. This increase was largely the result of localised outbreaks in Bristol, Brighton, Manchester, and Cambridgeshire. Some outbreaks involved a substantial number of HIV positive men who have sex with men (MSM). The re-emergence of syphilis in the HIV era is a particular cause for concern, as genital ulcer disease is known to facilitate the spread of HIV infection. The public health response to these syphilis outbreaks has included health promotion campaigns, community based screening projects, and increased access to departments of genitourinary medicine.

Primary and secondary syphilis are diagnosed on the basis of symptoms and clinical signs, with confirmation by treponemal serology. Diagnosing syphilis in HIV co-infected patients can be difficult, particularly in individuals with low CD4 lymphocyte counts, because patients may present with atypical symptoms, such as herpetiform ulceration. Serological screening tests for syphilis include cardiolipin specific antibody tests, based tests (Venereal Disease Research Laboratory (VDRL) test and rapid plasma reagin (RPR) test) and treponemal specific antibody tests (Treponema pallidum haemagglutination assay (TPHA), T pallidum particle agglutination assay (TPPA)), fluorescent treponemal antibody test (FTA), or treponemal enzyme immunoassay (EIA), which detect IgG and/or IgM. The sensitivity of these tests varies for primary syphilis: VDRL/RPR (70–80%), TPHA/TPPA (70–80%), treponemal EIA (85–90%), and FTA-abs (85–90%). A serological response to the presence of T pallidum usually takes between 1–4 weeks to develop, and is normally present by the time the primary chance is seen, with the earliest serological detection being achieved by IgM specific EIA. However, serological screening tests may fail to detect up to 30% of primary syphilis.

Syphilitic ulceration, most commonly seen on the ano-genital skin or in the mouth, offers an opportunity for direct detection of T pallidum early in infection. In the United Kingdom, the only direct detection method used is dark ground microscopy (DGM), which has demonstrated a sensitivity of 79–97% and a specificity of 77–100% in previous studies. However, DGM requires levels of skill and experience that are not common in the United Kingdom, and this test is unsuitable for specimens from the mouth or rectum because non-pathogenic spirochaetes may exist at these sites. Direct fluorescent antibody (DFA) testing is an alternative to dark ground microscopy, but this is not available in the United Kingdom. Polymerase chain reaction (PCR) offers an attractive option for the direct detection of T pallidum and several protocols have already been described. The assays are pathogenic treponeme specific: 95–97%, so oral and rectal specimens may be tested; and sensitive: 91–95%, with a reported limit of detection as low as 1–65 organisms.

In response to the increase in infectious syphilis in the United Kingdom, the Genitourinary Infections Reference Laboratory (GUIRL) offered PCR testing for syphilis on a trial basis. The PCR developed by Orle et al was used because it has been widely used in clinical studies and, in its complete form, allows the detection of three pathogens that cause genital ulcers (Haemophilus ducreyi, herpes simplex virus (HSV), and T pallidum). In this paper we report the results obtained with the PCR test and compare them with the diagnoses given by the sending clinicians, which were made.
on the basis of their clinical information and laboratory test results.

**METHODS**

**Transport and storage of samples**
Swabs of ano-genital or oral ulcers that were suspected to be syphilitic were requested between March 2000 and September 2001. These were sent either in dry sterile containers or placed in vials containing 1–1.5 ml transport medium—for example, viral or chlamydia transport medium. Samples were posted to the GUIRL and refrigerated (4°C) upon receipt.

**Sample preparation**
Batches of samples were processed on a weekly basis. Dry swabs were hydrated with 1 ml sterile saline solution for 1–5 minutes. All swabs were vortexed briefly before the saline solution or transport medium was transferred to sterile 1.5 ml tubes and centrifuged (13 000 g) for 1 minute. The top 0.8–1.3 ml of supernatant was removed and discarded leaving the remaining 200 µl supernatant and any cellular deposit for DNA extraction. A negative control sample (1 ml sterile saline) was included in every batch of samples. DNA extraction was carried out using the QIAamp DNA Blood mini kit (Qiagen Ltd, Crawley, West Sussex, UK) using the blood and body fluid spin protocol. DNA was eluted in 150 µl sterile distilled water, and a 25 µl aliquot was used for the PCR assay.

**PCR and detection**
The primers and thermal cycling conditions from the genital ulcer disease multiplex PCR were used. This amplifies a 260 bp region of the 47 kDa integral membrane lipoprotein gene using the following primers: KO3A 5’ GAAGTTTGTCCCAGTTGCGGTT and KO4 5’CAGAGCCATCAGGCTTTTTCA. Each reaction contained 0.5 µM primers KO3A and KO4, 1× PCR buffer (50 mM KCl, 20 mM TRIS-HCl pH 8.4), 1.5 mM MgCl2, 0.2 mM dNTPs, and 1.25 units of Platinum DNA polymerase (Invitrogen Life Technologies, Paisley, UK). Included in each PCR run were a negative control sample (25 µl distilled water), a positive control sample (distilled water containing 100 pg T. pallidum DNA), and an inhibition control for each sample (25 µl sample spiked with 100 pg T. pallidum DNA). Amplification was carried out on a Perkin Elmer 9700 using the 9600 ramp setting and the published thermal cycling conditions, which were as follows: 95°C for 2 minutes, then 35 cycles of 95°C for 20 seconds, 62°C for 20 seconds, and 72°C for 20 seconds. PCR products were analysed by electrophoresis using a 2% agarose gel.

**Control DNA**
Using standard methods, genomic DNA was extracted from purified T. pallidum Nicols strain (Newmarket Laboratories Ltd, Newmarket, UK) and quantified by ultraviolet spectrophotometry. A serial dilution of purified T. pallidum DNA was used to determine the detection limit of the PCR.

**Clinical details and laboratory test results**
Demographic and clinical details and relevant laboratory test results were obtained retrospectively by questionnaire. Data requested included the date and results from DGM (if performed) and serological tests, details of any antibiotic treatment before obtaining the swab sample, the HIV status of the individual and their sexual orientation. Clinicians responding to the questionnaire were asked to classify their diagnosis of each case as primary syphilis, secondary syphilis, or “not syphilis” according to all the available test results and to indicate if an alternative laboratory diagnosis was made—for example, an HSV infection. The particular laboratory tests used varied according to local diagnostic practices. The serological tests used included RPR, VDRL, TPHA, TPPA, an EIA detecting IgM and IgG, and an EIA detecting IgM only.

**RESULTS**

**Demography**
Samples were submitted from 117 patients, 98 of which are analysed below (see table 1). Data from 14 patients were incomplete (no questionnaire was returned) and excluded from this study. Samples from three patients were not from ulcers and were excluded from further analysis; two were swabs of skin rashes (on the foot and chest) and one was a swab of a sacral abscess (although these sites are suitable for sampling, a scraping rather than a swab is required to ensure an adequate sample is provided). After analysis of the serological data, samples from a further two patients were excluded: one patient was diagnosed clinically as having primary syphilis, with no confirmatory serology; the second was diagnosed with early latent syphilis and therefore by definition any ulcer sampled (in this case a mouth ulcer) would not be caused by T. pallidum.

The majority of patients had attended GUM clinics in the Greater Manchester area (70) with others attending clinics in London (19), Poole (five), Bristol (two), Dublin (one), and Worcester (one). Most patients (86) were male and, of these, a majority were MSM (58). Of the MSM, 24 were known to be HIV positive, with a mean CD4 lymphocyte count of 333 x 10^3 µl (range 65–641, normal range 500–1500; the count for two of these patients was unknown). Nine were receiving highly active antiretroviral treatment.

**PCR testing**
Swab specimens were obtained from the following sites: penis (47); oral cavity, tongue and lips (25); anus (18); vulva (12): rectum (three); and no site was specified for eight ulcer swabs. Duplicate samples (either two swabs of different ulcers or duplicate swabs taken on sequential clinic visits) were submitted from 13 patients. Three swab samples were submitted from one patient. Samples from these 14 patients represented 16 episodes of ulceration (two patients presented with two separate infections during the 19 month period of this study). One sample per episode of ulceration was included in the analysis. For six patients both samples were positive by PCR and for five patients both samples were negative by PCR.

One patient diagnosed with primary syphilis had a PCR positive sample at presentation and a PCR negative sample a week later, following treatment with procaine penicillin; these samples represent a single episode of infection and only the first sample was included in the analysis. Another patient, diagnosed with primary syphilis, had a PCR positive penile ulcer sample at presentation and was treated with a 17 day course of procaine penicillin; 2 days after completion of treatment, he presented with a peri-anal ulcer that was PCR negative but HSV culture positive. These two PCR samples represent two different episodes of ulceration (although it is not known whether the HSV infection was newly acquired or recurrent) and both were included in the analysis.

One patient presenting with a penile ulcer, had negative serology and a negative PCR test on the first visit and was diagnosed as “not syphilis,” but 5 months later presented with penile and oral ulcers, both PCR positive, and had positive treponemal serology consistent with secondary syphilis. These three samples represent two episodes of ulceration; the first sample and only one sample from the second visit were included in the analysis. It is possible that
the first presentation was primary syphilis before seroconversion, although this cannot be proved.

**PCR detection limit**

The detection limit of the PCR was determined as 1 pg *T. pallidum* DNA representing approximately 800 genome copies. All negative DNA extraction and PCR controls included in each batch of samples were negative and all positive PCR controls were positive. Inhibition tests of all samples revealed that there was no inhibition of the PCR reaction.

**Clinical diagnosis and laboratory test results**

Table 1 summarises details of serological tests, DGM and PCR, with patients grouped according to the diagnosis given by the sending clinician on the basis of clinical details and laboratory test results. Twenty nine cases of syphilis were identified by serology; 19 were defined as primary and 10 as secondary syphilis. Only five of 19 primary cases had a serological test to detect IgM alone; four were IgM positive and the fifth, who had no detectable anti-treponemal IgM, was a re-infection of a case previously identified as secondary syphilis in 1992 and subsequently treated. DGM was carried out on specimens from 13 patients with syphilis and was positive for two of the primary cases. PCR was carried out on specimens from all 30 cases of syphilis and was positive in 26 cases (18 primary and eight secondary cases). Two HIV positive patients (one with a CD4 lymphocyte count of 350, the count for the second was unknown) tested PCR positive 12 and 21 days, respectively, before syphilis seroconversion.

There were 71 patients who were diagnosed as not having syphilis, including 17 cases for whom a laboratory confirmed diagnosis of HSV was given and six with a previous history of treated syphilis. DGM was carried out on specimens from 21 of 71 patients, 20 of which were negative. One specimen, an anal swab, was positive, but the serology for this patient (RPR, TPHA, and an IgG+IgM EIA) was negative on two occasions, hence the clinician’s classification of this case as “not syphilis” (the PCR result for this patient was negative).

**Discrepancies between serological and PCR results**

One case of primary syphilis (two samples from this patient, one penile and one mouth ulcer swab) and two cases of secondary syphilis (two penile swabs and one mouth swab) were PCR negative.

One case, diagnosed as “not syphilis” on the basis of consistently negative treponemal serology (RPR, TPHA, and EIAs for IgM only and IgM+IgG combined were negative on three occasions) had a PCR positive penile swab. Only two other samples were processed alongside this sample in the laboratory, neither of which were PCR positive. This patient was HIV positive with a CD4 lymphocyte count of 180 (normal range 500–1500) and, coincidentally, had received a course of co-amoxiclav 6 days after the PCR positive swab was taken.

**Performance of the PCR test**

The sensitivity, specificity, positive and negative predictive values for the PCR were calculated as 94.7%, 98.6%, 94.7%, and 98.6%, respectively, for primary syphilis and 80%, 98.6%, 88.9%, and 97.2%, respectively, for secondary syphilis.

**DISCUSSION**

The epidemiology of syphilis in the United Kingdom has undergone significant changes recently. Localised outbreaks
have resulted in many patients presenting with ulcerative lesions suitable for direct detection of *Treponema pallidum* by PCR, in sites where DGM cannot be used (mainly oral lesions).

In response to the changing epidemiology of syphilis, we have offered a *T pallidum* specific PCR assay as a trial diagnostic service. Over the 19 month period of this study, swabs from 117 patients were submitted for syphilis PCR, indicating that a demand for the service existed, at least in some areas where syphilis was prevalent (principally Manchester and London).

Data presented in table 1 demonstrate that for the majority of cases the PCR results correlate well with the serology obtained: 18/19 primary and 8/10 secondary cases of syphilis were detected by PCR. Two HIV positive patients were positive by PCR more than 1 week before treponemal seroconversion.

The PCR successfully detected *T pallidum* from ulcers present during primary or secondary syphilis from oral, genital, and anal sites. These data served to confirm positive cases or, on occasion, to pre-empt the serological results. Negative PCR results were also of value, allowing the prompt elimination of syphilis from the diagnosis in many instances. HSV infection was a frequent diagnosis among those cases that were identified as “not syphilis,” which suggests that a PCR offering a differential diagnosis between *T pallidum* and HSV would be a valuable tool in regions of the United Kingdom where syphilis outbreaks are ongoing. This is possible if the multiplex ulcer PCR9 is used in its entirety, and it would also detect the occasional *H ducreyi* infection.

There were four discrepancies between diagnoses given and the PCR results. Three patients with serologically proved syphilis gave false negative PCR results: these may represent sampling ulcers of non-treponemal origin in patients with coincident positive treponemal serology or simply reflect the limited sensitivity of the PCR in the simplified format used here. Ulcer specimens typically contain 22×10³–5.7×10⁶ organisms11; hence a detection limit of 1 pg DNA (equivalent to approximately 800 organisms) should have been adequate. However, the number of organisms present in resolving ulcers may be small and sampling technique may vary. A PCR with a lower limit of detection could be achieved either by using the enzyme linked oligonucleotide sorbent assay to detect the PCR product (as advocated in the original paper1) or by converting the PCR to one of the real time PCR platforms (Light cycler or Taqman). The final discrepant result was a false-positive PCR result in a patient with consistently negative syphilis serology. Unfortunately, several DNA samples (including this one) were lost to the study and so it was not possible to carry out discrepant analysis using an alternative *T pallidum* PCR. One possibility for this discrepancy is that prompt antibiotic treatment (coincidental) coupled with the patient’s immune dysfunction may have blunted a serological response to *T pallidum*. Much of the syphilis literature relies on diagnosis by serology, hence serologically negative cases of syphilis in patients with immune dysfunction may be underestimated.

DGM was used infrequently, in only 34 of 84 suitable samples. This finding is not surprising, as generations of UK trained genitourinary physicians have seen little if any syphilis and have not acquired the expertise necessary for diagnosis using DGM. There is a dominance of samples from oral and anal sites in outbreaks of syphilis that occur within the “gay” community (as in Manchester and Brighton). Although DGM can be carried out on anal ulcers, it is not advised for oral ulcers because they usually harbour other spirochaetes.

The lack of use of specific anti-treponemal IgM testing in primary syphilis (used in only five of 19 cases) is surprising as this test has a sensitivity in excess of 80%,13 this finding also suggests lack of compliance with UK guidelines for the serological diagnosis of syphilis, which recommends specific IgM testing in suspected primary infection.12,13 Diagnostic methods that can aid rapid identification of the disease can, by expediting treatment, reduce onward transmission. A PCR test for syphilis would offer a direct detection method that is suitable for ulcer specimens from all body sites, including the mouth and rectum. PCR may provide earlier diagnosis for those patients who present with an ulcer in the serological “window period,” for patients who refuse venepuncture (one in this study), and for those who fail to attend for sequential serological analysis. A diagnostic PCR service for syphilis could make a valuable contribution to the diagnosis of other sexually transmitted infections. Moreover, the acquisition of treponemal DNA from PCR positive samples could provide a valuable resource for molecular epidemiology.14 This could facilitate epidemiological investigation of syphilis, a re-emerging infection in the United Kingdom.

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**CONTRIBUTORS**

This work was initiated by HMP and AJH; PCR assays were carried out by HMP, SPI and MK were major providers of clinical material and details; preparation of the manuscript was carried out by HMP, SPI, AJH, and MK.

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