Simplified PCR for Detection of *Haemophilus ducreyi* and Diagnosis of Chancroid

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A simplified PCR was developed for detection of *Haemophilus ducreyi* in samples from chancroid patients. The strategy included a straightforward chloroform extraction sample preparation method, a one-tube nested PCR to minimize contamination risks, and a colorimetric method for detection of products. Primers were designed from published nucleotide sequences of the 16S rRNA gene of *H. ducreyi*, with longer outer primers for annealing at a higher temperature and shorter inner primers labelled with biotin and digoxigenin for binding with avidin and colorimetric detection. The PCR technique detected all 35 strains of *H. ducreyi* tested, from four different geographical regions, and was negative for other, related strains of bacteria and for the common contaminating bacteria tested. Of 25 samples from *H. ducreyi* culture-positive chancroid patients, 24 were PCR positive and 1 produced a weak reaction. Of 83 samples from clinical cases of chancroid in the Republic of South Africa, 69 were PCR positive. The sensitivity of PCR compared with that of clinical diagnosis was 83%. All 50 negative control samples were negative. Encouraging results were also obtained with a consecutive series of 25 genital ulcer patients in Tanzania, of whom 9 were PCR positive. The adaptations of this simplified PCR strategy, at the sensitivity and specificity levels obtained, mean it will be useful for detection of *H. ducreyi* in areas where the organism is endemic, particularly where testing by culture is difficult or impossible.

Chancroid, a genital ulcer disease caused by *Haemophilus ducreyi*, is prevalent mainly in developing countries, although a fourfold increase in incidence was reported in the United States between 1980 and 1991 (8). Detection and treatment have become increasingly important, as genital ulcer disease has been strongly associated with an increased risk of transmission of human immunodeficiency virus infection (1, 18, 19).

H. ducreyi is a fastidious organism and requires complex media and growth conditions for culture, so that even in experienced and well-equipped laboratories it can be isolated from only 50 to 90% of cases of clinically diagnosed chancroid. In inexperienced laboratories, the isolation rate is less than 50% (5, 16). In many areas, diagnosis is based on the clinical picture, the sensitivity of which depends on the prevalence of chancroid in the community and is reported to be, at best, between 75 and 80% (4, 6). An alternative method to improve diagnosis and detect asymptomatic carriage would be an important advance.

Several such methods have been reported, including direct examination by Gram stain, which has been shown to be less than 50% sensitive (15), use of monoclonal or polyclonal antibodies in immunofluorescence tests (7, 12), and use of DNA probes (17). None of these methods is widely used for clinical diagnosis, since these techniques have not been sufficiently sensitive or specific. However, the advent of newer DNA amplification methods using the PCR offer a simpler and more sensitive and specific approach to diagnosis. A PCR-based technique for detection of *H. ducreyi* has been reported by Chui et al. (3). Their method gave 83 to 98% sensitivity and 51 to 67% specificity compared with culture. Another was reported by Johnson et al. (11), who reported only 62% sensitivity; the low value, they think, was due to *Taq* DNA polymerase inhibitors.

We have adapted a simplified strategy for PCR, originally developed by Wilson et al. for detection of *Mycobacterium tuberculosis* (23), to the detection of *H. ducreyi* in clinical samples. Standard PCR methods are not easily applicable in the field, but this simplified strategy allows more samples to be processed without the need for equipment and expertise to run and read gels. The method was first evaluated with pure cultures of *H. ducreyi*; other, related organisms; and common contaminating organisms of the genital tract and then with culture-positive clinical samples, samples from patients with clinically diagnosed chancroid, negative control samples, and samples from unselected genital ulcer patients in Tanzania.

MATERIALS AND METHODS

Strains of *H. ducreyi*. We used 35 strains of *H. ducreyi*. All were isolated from clinical cases of chancroid, 12 strains were from Thailand, 12 were from the Republic of South Africa, 7 were from The Gambia, and 4 were from Tanzania. In addition, a dilution series was prepared with two different strains to assess the sensitivity of the assay.

Strains of other organisms. A series of 15 species were selected on the basis that they are closely related species or occur as common contaminants of the genital tract. Strains were from the National Collection of Type Cultures or from the London School of Hygiene and Tropical Medicine culture collection. The species and strains tested were as follows; *H. influenzae* NCTC8466, NCTC8468, and NCTC8469; *H. parahaemolyticus* NCTC8479; *H. parainfluenzae* NCTC4101 and NCTC7857; *H. parasuis* NCTC4557; *Pasteurella multocida* NCTC948 and NCTC8771; *P. urea* NCTC 10219; *Gardnerella vaginalis* NCTC 10287 and LSHTM1-5; *Escherichia coli* LSHTM232 and NCTC10418; *Staphylococcus aureus* NCTC6571 and NCTC8532; *Streptococcus faecalis* NCTC8213; *S. faecium* NCTC7171; *Corynebacterium ulcerans* NCTC7908; *Bacillus subtilis* NCTC3610; *Neisseria gonorrhoeae* NCTC10022 and LSHTM/TZ1 to LSHTM/TZ10; and

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Chlamydia trachomatis L1, L2, F, and K (London School of Hygiene and Tropical Medicine culture collection).

Clinical samples. Genital ulcer swabs were collected from 25 *H. ducreyi* culture-positive genital ulcer patients attending a sexually transmitted disease clinic in Mwanza, northern Tanzania. These were stored at −20°C, transported to London, England, on dry ice, and used as positive controls. Genital ulcer swabs were taken from 83 patients with a clinical diagnosis of chancroid at the University Hospital in Johannesburg, Republic of South Africa. These were also cultured for *H. ducreyi* in Johannesburg, Republic of South Africa. The results were not made available until the PCR was completed. Samples were stored at −70°C and transported to London, England, on dry ice. Cervical swabs were collected from 50 antenatal clinic attenders at University College Hospital, London, England, and used as negative controls. Genital ulcer swabs were taken from 25 consecutive genital ulcer patients attending a sexually transmitted disease clinic in Mwanza, Tanzania. These were cultured for *H. ducreyi* locally, but the results of culture were not made available until the PCR was completed. Swabs were stored at −20°C and transported to London, England, on dry ice.

Swabs for PCR were always taken after the swabs for culture and were placed in 0.5 ml of phosphate-buffered saline for storage. All swabs were processed at the London School of Hygiene and Tropical Medicine.

Processing of samples. Samples were defrosted, and each swab was squeezed on the side of the tube to expel its contents; the swab was then removed. The sample remaining in the tube was then divided into two for duplicate processing and analysis. The samples were centrifuged at 12,000 × *g* for 5 min. Supernatants were discarded, and the pellets were resuspended in 1 ml of 50 mM Tris buffer, pH 8.3. They were centrifuged again at 12,000 × *g* for 5 min, supernatants were discarded, and the pellets were resuspended in 50 μl of Tris buffer, pH 8.3. Samples were heat treated at 80°C for 30 min to inactivate any live bacteria or viruses present. After the samples had cooled, 50 μl of chloroform was added and mixed well and the samples were centrifuged at 12,000 × *g* for 2 min. A 5-μl volume of the upper aqueous phase containing the nucleic acids was used in a 20-μl PCR. If stored prior to use, samples were stored at −20°C without removal of the lower chloroform layer. After storage and defrosting, samples were recentrifuged prior to analysis.

PCR. The one-tube nested PCR described by Wilson et al. was used (23). The PCR was carried out in 0.5-ml microcentrifuge tubes with a Hybaid Combi TR3 Thermal Reactor (Hybaid Ltd., Teddington, England). The PCR target was the 16S rRNA gene of *H. ducreyi*, and the primer sequences were taken from those published by Rossau et al. (21).

The outer primers were HD996 (5′-TGTGACAGGTGCTGCATGGCTGTC) and HD1400 (3′-GCGATTACTAGCGATTCCGACTTCA). The inner primers were HD1279 (3′-GTCGAGTTGCAGACTC) and HD1078 (5′-ATGTAA/GT GATGGAAC). (A/G signifies that the primer contained either base at this position.) A batch of inner primers, HD1279 and HD1078, labelled at their 5′ ends with biotin and digoxigenin, respectively, during their commercial synthesis (R and D Systems Europe Limited), were also prepared. Primers were used at concentrations of 0.6 pmol of each outer primer and 10 pmol of each inner primer per 20-μl reaction mixture. The inner primers were used as a 2:1 ratio of unlabelled to labelled primer.

Reaction and cycling parameters were as described for the PCR used for diagnosis of tuberculosis (23). Briefly, the one-tube nested PCR is designed to enable a nested PCR to be performed automatically by the thermal cycler. The PCR is performed in two consecutive phases of 30 and 20 cycles. During the first 30 cycles, performed at a high annealing temperature of 65°C, a product is produced from the outer primers. During the subsequent 20 cycles at a low annealing temperature of 50°C, the product from the outer primers acts as a target for the inner primers and the smaller, nested product is generated.

Colorimetric detection of PCR product. During the second PCR phase, the labelled inner primers become incorporated into the PCR product so that the product has a biotin label and a digoxigenin label. This enables any product to be detected by capture in avidin-coated microtiter plates. Subsequent detection is done with an anti-digoxigenin antibody–alkaline phosphatase conjugate.

Microtiter plates were coated with avidin (10 μg/ml in 50 mM carbonate buffer, pH 9.6) for 1 h at 37°C and washed twice with TBS-Tween 20 (Tris-buffered saline [pH 7.5] plus 0.5% Tween 20). A 250-μl volume of TBS-Tween 20 was added to each reaction tube, and the tube was heated to 68°C to melt the wax pellet and allow the contents to mix. These reaction mixtures were assayed in duplicate. A 100-μl volume of the diluted product was added to each well, and the plates were left at room temperature for 30 min to allow capture of the biotinylated product. The plate was washed twice in TBS-Tween 20, and 100 μl of anti-digoxigenin antibody–alkaline phosphatase conjugate (Boehringer Mannheim) diluted 1/5,000 in TBS-Tween 20 was added to each well. The plate was left at room temperature for 30 min, and unbound conjugate was removed by washing the plate four times with TBS-Tween 20 and twice with TBS. A 100-μl volume of substrate (1 mg/ml of *para*-nitrophenol in 50 mM carbonate buffer [pH 9.6]–1 mM magnesium chloride) was added to each well. After 30 min, plates were read by visual inspection or on an enzyme-linked immunosorbent assay (ELISA) reader at an optical density of 405 nm. Negative PCR controls were used as negative ELISA controls; similarly, *H. ducreyi*-positive samples were used as positive controls. Wells with no addition of PCR products were used to blank the ELISA reader. All samples were regarded as negative below an ELISA
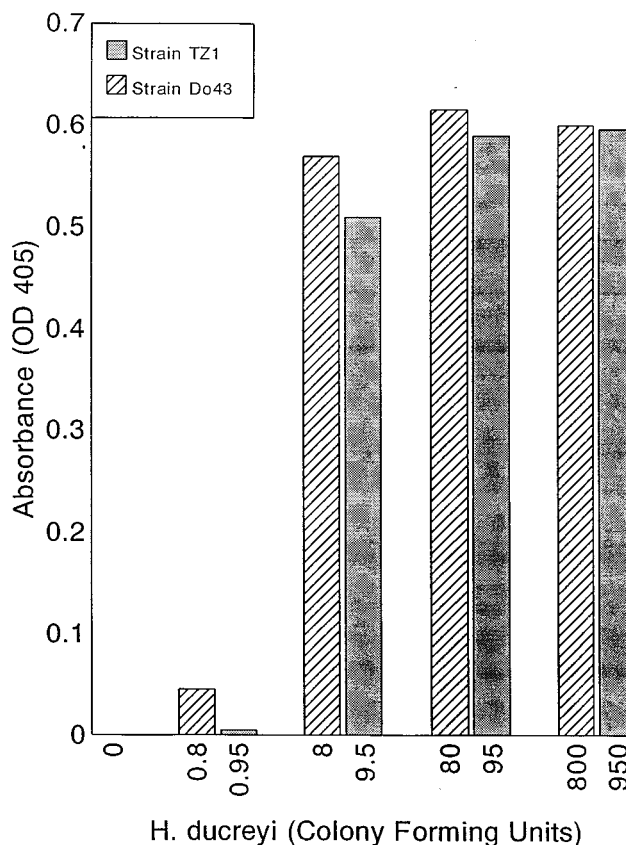

FIG. 1. Sensitivity of colorimetric one-tube nested PCR for detection of *H. ducreyi*.

reading of 0.100 and positive above a reading of 0.250. Intermediates were designated inconclusive.

RESULTS

Sensitivity and specificity. All *H. ducreyi* cultures tested by PCR gave a strong positive color reaction. All strains of *H. influenzae*, *H. parahaemolyticus*, *H. parainfluenzae*, *H. parasuis*, *P. multocida*, *P. urea*, *G. vaginalis*, *E. coli*, *S. aureus*, *S. faecalis*, *S. faecium*, *C. ulcerans*, *B. subtilis*, *N. gonorrhoeae*, and *C. trachomatis* were negative.

Titrations were performed on two strains of *H. ducreyi*. These were grown for 24 h, and the growth was then suspended in phosphate-buffered saline in Bijou bottles half filled with small glass beads. These were vigorously vortexed to break up the clumps and then allowed to stand for 10 min, and the supernatant was taken for use in the PCR and for viable counting. The results are illustrated in Fig. 1. Approximately 1 CFU per PCR gave a very weak color reaction, whereas approximately 10 CFU per PCR gave a strong color reaction which was clearly detectable.

ELISA results. Color detection gave clear-cut results. All negative controls and samples had readings well below the cutoff point (optical density of 0.100). Positive controls and positive samples were above an optical density of 0.250. A weak reaction (optical density of 0.100 to 0.250), occurred with only four samples. This paler color reaction could be detected by eye and was designated inconclusive.

Positive control patient samples. Of the 25 samples from culture-positive cases in Tanzania, 24 were positive by PCR.

TABLE 1. PCR results of samples from clinical chancroid patients from the Republic of South Africa

PCR result	No. of culture-positive samples	No. of culture-negative samples	Total no. of samples
Positive	59	10	69
Negative	4	6	10
Inconclusive	1	3	4
Total	64	19	83

With the remaining sample, both duplicates gave a very weak color reaction on primary and repeat testing and were classified as inconclusive.

Chancroid patient samples. Of the 83 samples from clinically diagnosed chancroid patients from South Africa, 69 were *H. ducreyi* PCR positive, 10 were PCR negative, and 4 were considered inconclusive because there was either a very weak color reaction (3 samples) or a discrepancy between the duplicates tested (1 sample), even after repeated testing. Culture results, obtained independently, showed that *H. ducreyi* was isolated from 64 samples and 19 were negative (Table 1). Discrepancies occurred with 4 samples which were culture positive but PCR negative and 10 samples which were PCR positive but culture negative. The PCR was repeated with these samples, and the results remained unchanged. The sensitivity of the PCR procedure compared with clinical diagnosis was 83%, and culture sensitivity was 77%.

Negative control patient samples. The 50 cervical samples taken from women attending the University College Hospital clinic in London, England, were used as negative controls, and these samples were interspersed among the test samples. All of these control samples were PCR negative.

Genital ulcer patient samples. Of the 25 samples processed, 9 were PCR positive, 16 were PCR negative, 4 were culture positive, and 21 were culture negative (Table 2). Discrepancies occurred with five samples which were culture negative but PCR positive.

DISCUSSION

The PCR strategy described here was chosen as the simplest test to perform, with minimum opportunity for errors and contamination. This should promote its use in areas of the world where chancroid is prevalent but technology and finances are limited. The one-tube nested PCR has been successfully used for other detection systems, for example, for tuberculosis (23), and color detection methods have also been successfully used to detect chlamydiae (10).

Diagnostic tests based on the 16S rRNA gene are likely to be highly sensitive because of the gene's high copy number. Although highly conserved among eubacteria, it contains species-specific regions from which the sequences of our primers were selected (2, 9, 21, 22).

Colorimetric detection of products simplified detection and eliminated the need to run and visualize electrophoretic gels, which minimizes the equipment required. The technique has been shown to be as sensitive and specific as agarose gel electrophoresis in detecting PCR products (23). The resulting color reaction could be read clearly by eye, which eliminates the need for an ELISA plate reader.

Although there is no typing system for *H. ducreyi*, strains isolated have different growth characteristics and nutritional requirements (13). To investigate any geographical strain differences, the PCR technique was used with strains from four areas: eastern, western, and southern Africa and Asia. There were no differences in the performance of the test, which detected all strains with similarly high sensitivity. All other *Haemophilus* species, members of the family *Pasteurellaceae*, and common contaminating or genital organisms were negative, making the test highly specific for *H. ducreyi*.

Of the 25 culture-positive clinical samples from Tanzania first tested, 1 gave a very weak PCR and could not be classified as positive. Culture results for this sample showed that very few *H. ducreyi* organisms (fewer than 20) were isolated. In this case, it is likely that the second swab, used for PCR, failed to pick up sufficient organisms for detection.

Of the samples from clinically diagnosed chancroid patients from the Republic of South Africa, four were culture positive but PCR negative. This was shown not to be due to inhibition of the PCR (results not shown), but again this could be due to low numbers of organisms on the second swab used for PCR. Conversely, there were 10 PCR positives which were culture negative; this is more understandable, as there are known limitations to the culture system (5). Compared with that of clinical diagnosis, the sensitivity of the PCR was 83%.

The group of patients from the University College Hospital were chosen as negative controls. It has been suggested that asymptomatic carriage of *H. ducreyi* may occur in populations in areas where the organism is endemic (14, 20). Gynecological patients in London, England, are unlikely ever to have been in contact with *H. ducreyi* but are likely to harbor commensal organisms similar to those found in African women. All of these samples were clearly negative.

The results of the genital ulcer study in Mwanza, Tanzania, were encouraging. There were five culture-negative, PCR-positive samples; two of these were consecutive samples from the same patient whose ulcer was not cured by first-line treatment, which adds credence to the PCR result. It is also known that the culture system used here is not optimal, with some problems of contamination (personal communication), and could easily miss positive samples. The PCR is being further evaluated and used in a larger study of the etiology of genital ulcers in this area.

Although the methods for the PCR have been simplified for field use, the problems of contamination will persist, particularly if reasonable precautions are not taken. Reaction mixtures should be prepared in a clean area, away from samples and from product analysis (23). Throughout the evaluation period, sporadic low-level contamination did occur, manifesting itself as unmatched duplicate samples. These problems were resolved in all cases on reinvestigation of the stored duplicate extracts by PCR.

The high sensitivity and specificity of this simplified colorimetric PCR means that it will be useful for the diagnosis of *H. ducreyi* infection, particularly in the larger centers, where new technology is affordable or available. It could also prove to be useful in epidemiological studies, especially where samples

TABLE 2. PCR results of samples from genital ulcer patients from Tanzania

PCR result	No. of culture-positive samples	No. of culture-negative samples	Total no. of samples
Positive	4	5	9
Negative	0	16	16
Total	4	21	25

need to be taken in the field and in areas where culture is difficult or impossible, for example, in screening large numbers of potential asymptomatic carriers to elucidate their role in the transmission of chancroid.

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