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Development of the Polymerase Chain Reaction for Diagnosis of Chancroid

LINDA CHUI,[1] WILLIAM ALBRITTON,[1]* BRUCE PASTER,[2] IAN MACLEAN,[3] AND RAYMOND MARUSYK[1]

Provincial Laboratory of Public Health, University of Alberta, Edmonton, Alberta T6G 2J2,[1] Canada; Forsyth Dental Center, Boston, Massachusetts 02115[2]; and Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 2N2,[3] Canada

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The published nucleotide sequences of the 16S rRNA gene of *Haemophilus ducreyi* were used to develop primer sets and probes for the diagnosis of chancroid by polymerase chain reaction (PCR) DNA amplification. One set of broad specificity primers yielded a 303-bp PCR product from all bacteria tested. Two 16-base probes internal to this sequence were species specific for *H. ducreyi* when tested with 12 species of the families *Pasteurellaceae* and *Enterobacteriaceae*. The two probes in combination with the broad specificity primers were 100% sensitive with 51 strains of *H. ducreyi* isolated from six continents over a 15-year period. The direct detection of *H. ducreyi* from 100 clinical specimens by PCR showed a sensitivity of 83 to 98% and a specificity of 51 to 67%, depending on the number of amplification cycles.

The accuracy of the clinical diagnosis of genital ulcer disease as chancroid has been reported to be 75 to 80% at best (6, 10). Both false-positive and false-negative clinical variants of chancroid have been reported (2, 16, 20, 29, 32).

The sensitivity of culture for the confirmation of clinical disease has been improved in recent years to 50 to 90% in experienced laboratories in areas where chancroid is endemic (9, 31). Culture sensitivity in inexperienced laboratories during epidemics has been reported to be less than 50% (24). Efficient transport media for *Haemophilus ducreyi* that would allow culture diagnosis in distant laboratories with prolonged transportation times have not been reported. For these reasons, there has been considerable interest in nonculture methods for the diagnosis of chancroid.

Direct examination of ulcer material by Gram's stain (3, 14) and electron microscopy of biopsy material (19) have both been suggested as diagnostic tests for chancroid. Recent studies have shown the sensitivity of the Gram stain to be less than 50% (5, 23), and the morphology of *H. ducreyi* is similar to those of many organisms found in the polymicrobial flora of most genital ulcers (4). Improvements in reagents for direct examination by incorporating adsorbed polyclonal (7, 11) and monoclonal (11, 12, 15, 30) antibodies into immunofluorescence tests have been reported. These reagents have not been widely used in clinical studies to establish their performance characteristics under routine use conditions.

With the introduction of DNA diagnostic methods (17) and the ability to amplify the signal with the polymerase chain reaction (PCR), newer tests should improve the sensitivity and specificity of diagnostic tests for chancroid. First-generation DNA probes for *H. ducreyi* have been reported with sensitivities of 10^3 to 10^4 organisms and 100% specificity (25, 26). We report here our results with the development of primer sets and probes for the diagnosis of chancroid by DNA amplification using PCR.

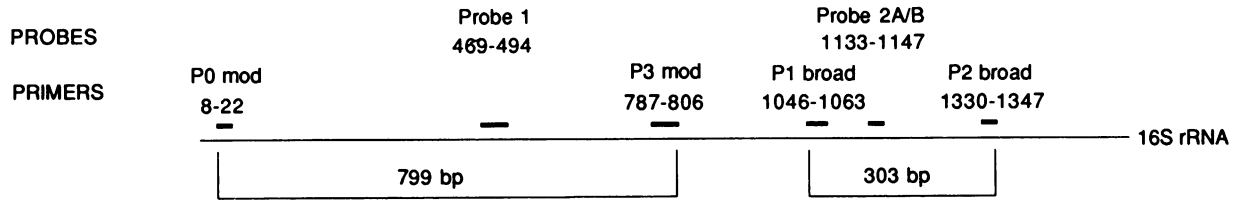
* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. Fifty-one strains of *H. ducreyi*, including the type strain CIP542, isolated from six continents over a 15-year period were selected for assessing sensitivity. The following twelve species from the families *Pasteurellaceae* and *Enterobacteriaceae* were selected for assessing specificity on the basis of rRNA sequence homology or because they are commonly isolated from genital ulcers: *Haemophilus paraphrophilus* ATCC 29241, *Haemophilus parainfluenzae* NCTC 7857, *Haemophilus haemolyticus* NCTC 10659, *Haemophilus* spp. (minor group 202), *Pasteurella ureae* NCTC 10219, *Actinobacillus pleuropneumoniae* ATCC 27088, *Actinobacillus equuli* NCTC 8529, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* LT2, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

Nucleic acid extraction. Bacterial colonies were scraped from plates with cotton swabs and washed twice with 12 mM Tris-HCl, pH 7.6. Cells were standardized to an optical density of 0.12 at 540 nm in the same Tris buffer. One milliliter of the standardized suspension was pelleted and resuspended in 100 μl of Tris buffer. Crude DNA was obtained by subjecting the cell suspension to three cycles of boiling and freezing. Ten microliters of each sample was used for PCR analysis.

Clinical specimens were taken in the Special Treatment Clinic in Nairobi, Kenya, from males with genital ulcers consistent with a clinical diagnosis of chancroid. The swabs were transported to the laboratory in Nairobi at ambient temperature in 1 ml of phosphate-buffered saline (PBS) containing chenodeoxycholate (Sigma) at 1 mg/ml and heated at 100°C for 15 min on arrival. Samples were stored at −70°C until transported to Edmonton, Canada. The method of extraction of nucleic acid from clinical specimens for PCR was adapted from Pollard et al. (27). A positive control containing 10^5 *H. ducreyi* organisms in chenodeoxycholate and a negative control of chenodeoxycholate were carried through each extraction procedure. Lysis buffer (200-μl 0.1 M Tris-HCl [pH 8.0], 0.1 M NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate [SDS], 50 μg of proteinase K per ml) was added to an equal volume of specimen and incubated at 37°C



P0 mod	5'- AGAGTTTGATC(AC)TGG -3'
P3 mod	5'- GGACTACCAGGGTATCTAAT -3'
P1 broad	5'- AGGTGCTGCATGGCTGTC -3'
P2 broad	5'- CTAGCGATTCCGACTTCA -3'
Probe 1	5'- CTGTGACTAACGTCAATCAATTTTGC -3'
Probe 2A	5'- ATGTAGTGATGGGAAC -3'
Probe 2B	5'- ATGTAATGATGGGAAC -3'

FIG. 1. Physical map and location of the oligonucleotide primers and probes within the 16S rRNA gene sequence. The sizes of the PCR amplification products are shown in the boxes. The nucleotide positions are those of Dewhirst et al. (8). P3 mod, P2 broad, and probe 1 are complementary to the published sequence.

for 2 h before extraction with phenol, phenol-chloroform, and chloroform. Nucleic acids were precipitated by addition of sodium acetate and ethanol and resolubilized in 100 μl of water.

An additional swab was taken and streaked onto a chocolated GC agar (Difco) plate supplemented with 5% (vol/vol) fetal calf serum and 3 mg of vancomycin per liter. The plate was incubated for 48 h at 33 to 35°C in a candle extinction jar for isolation of *H. ducreyi* (9, 24).

PCR. Two primer sets and two probes were selected from the *H. ducreyi* 16S rRNA sequence. These oligonucleotides were prepared by a PCR-MATE 391 DNA synthesizer (Applied Biosystems) according to the manufacturer's instructions. Ten microliters of the crude DNA extract or 50 μl of DNA extract from clinical material was added to a final volume of 100 μl of a PCR reaction mixture containing 30 pM (each) primer, 50 μM (each) deoxynucleotide triphosphates (Pharmacia), 1 U of *Taq* polymerase, and 1× reaction buffer (Bio/Can Scientific). The negative control containing no DNA and the positive control containing extracted DNA from *H. ducreyi* were included in all runs. Amplification of bacterial DNA was carried out in an automated thermal cycler (Perkin-Elmer Cetus) in which samples were denatured at 94°C for 1 min and then primer annealed at 40°C for 1 min and elongated at 72°C for 3 min. This cycle was repeated 25 times, and the amplified product was subjected to electrophoresis for 90 min at 160 V in 1% (wt/vol) agarose gels containing 0.05 μg of ethidium bromide per ml and visualized by UV illumination. DNA was transferred passively onto nylon membranes (Hybond; Amersham), UV cross-linked, and prehybridized at the hybridization temperature in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% (wt/vol) SDS, 5× Denhardt solution, and 0.5 mg of sheared denatured salmon sperm DNA per ml. The probes were end labelled with [^{32}P]ATP (Amersham) by using T4 polynucleotide kinase (Bethesda Research Laboratories) or with digoxigenin (Boehringer Mannheim) by using terminal transferase according to the manufacturer's instructions (Boehringer Mannheim). Hybridization was carried out at 65°C with probe 1, 43°C with probe 2A, and 40°C with probe 2B overnight in a shaking water bath. Washings for radioactive probes were done according to the method of Maniatis et al. (18), and washings for digoxigenin-labelled probes were done according to manufacturer's instructions. The blot was exposed to Kodak X-Omat AR film.

Sequencing of the PCR product. The amplified DNA product was excised and extracted with Geneclean (Bio 101). The purified DNA was sequenced directly with the dideoxytermination method, modified T7 DNA polymerase Sequenase (United States Biochemical), and the reagents provided in the kit. The samples were subjected to electrophoresis in a 6% (wt/vol) polyacrylamide–7 M urea sequencing gel, fixed, dried, and exposed to Kodak XAR-5 film.

Sensitivity of PCR primers. Cell pellets of *H. ducreyi* were washed and standardized to an optical density of 0.12 at 540 nm in 12 mM Tris-HCl, pH 7.6. Tenfold serial dilutions were made from 10^{-1} to 10^{-8}. The average number of CFU was determined by plating 100 μl of each dilution in triplicate. Crude DNA was extracted from an equal volume of each dilution, and 10 μl of the extract was used in the PCR reaction.

RESULTS

***H. ducreyi* primer sets and probes.** Two primer sets were evaluated for amplification of the 16S rRNA gene of *H. ducreyi* (Fig. 1). The choice of the 16S rRNA gene was based on multiple copies of the gene within the genome, the ability to use broad specificity primers for assessing amplification in culture-negative specimens, and the known sequence variability within eubacteria. The P0 mod and P3 mod primers have been described previously (34) and are similar to the broad specificity primers used by Weisburg et al. (33). The P1 broad and P2 broad primers were selected for the high level of sequence homology between published eubacterial 16S rRNA sequences. The probe 1 and probe 2 sequences were chosen from the known variable regions of published eubacterial 16S rRNA sequences and the published *H. ducreyi* sequence (8, 28). The probe 2A sequence was originally chosen from the sequence of Dewhirst et al. (8), and probe 2B was from the sequence of Rossau et al. (28) for the same region.

Sensitivity of *H. ducreyi* primers and probes. With the P0 mod and P3 mod primers, a strong 799-bp PCR amplification product was seen with only 48 of the 51 strains of *H. ducreyi* tested. The combination of P0 mod and P3 mod primers with probe 1 could detect only 10^5 to 10^6 organisms (data not

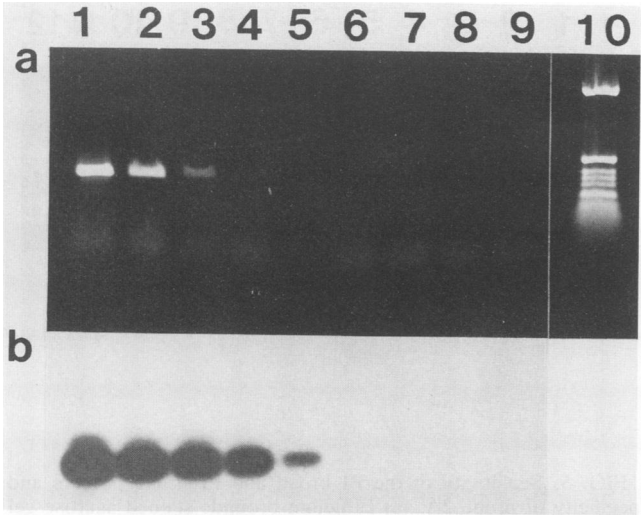


FIG. 2. Sensitivity of PCR detection of *H. ducreyi*. (a) Ethidium bromide-stained agarose gel showing the PCR products generated with the P1 broad and P2 broad primers for serial dilutions of *H. ducreyi* V1157. Lanes 1 to 8 contain approximately 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 organisms, respectively. Lane 9 is the negative control consisting of PCR reagents only. Lane 10 contains DNA size markers (pBR322 digested with *Hin*FI) 154 to 517 bp in size. (b) Autoradiogram developed after Southern transfer of the PCR products from panel a and hybridization with probe 2A.

shown). The P1 broad and P2 broad primers produced a 303-bp PCR product with all strains of *H. ducreyi* and all members of the *Pasteurellaceae* and *Enterobacteriaceae* families studied and with species commonly found in genital ulcers. The combination of P1 broad and P2 broad primers with probe 2A could detect 10^2 to 10^3 organisms after one round of 25 cycles of PCR (Fig. 2). When 25 μl of the amplification product from the first round of PCR was used as the template for a second round of 25 cycles of PCR, the sensitivity increased to 10^0 organisms (data not shown). However, beyond three rounds of 25 cycles, interference and a smearing of the amplification product in agarose gels were noted. Special precautions, such as aerosol-free pipette tips, were required to prevent contamination when reamplification was carried out, and 25 μl from the negative control of the previous round of amplification was always carried through the reamplification cycles.

Specificity of *H. ducreyi* primers and probes. To avoid the problem of false-negative results with clinical specimens (13), we deliberately chose primers with broad specificity. Probe 1 failed to hybridize to 3 of the 48 *H. ducreyi* amplification products with P0 mod and P3 mod primers (data not shown). Probe 2A hybridized strongly with 46 of the 51 *H. ducreyi* PCR amplification products with P1 broad and P2 broad primers. The failure of probe 2A to hybridize with the remaining five strains of *H. ducreyi* was investigated by sequencing the PCR product from strains that hybridized with and failed to hybridize with this probe (Fig. 3). Both strand sequences confirmed an additional G at position 1144, as reported by Dewhirst et al. (8), and there was a G:A transition at position 1137 in some strains (8, 28). A modified probe 2B was synthesized (Fig. 1), and this probe recognized all five strains that did not react with the probe 2A oligonucleotide (Fig. 4). Probe 2A or probe 2B oligonucleotides were 100% specific for *H. ducreyi* when hybridized with amplification products from 10 species of eubacteria (Fig. 5). A similar PCR amplification product which failed to hybridize with probes 2A and 2B was seen with *H. paraphrophilus* ATCC 29241 and *H. parainfluenzae* NCTC 7857 (data not shown). Thus, the combination of P1 broad and P2 broad primers with probe 2A or 2B was 100% sensitive and 100% specific for *H. ducreyi* with pure cultures of clinical isolates.

Detection of *H. ducreyi* in clinical specimens. Preliminary data (not shown) indicated that dry swabs or swabs placed in PBS alone were less satisfactory for PCR than swabs heated in chenodeoxycholate. Over a 6-month period, 100 specimens from patients with genital ulcer disease were processed by PCR and culture. Three specimens were contaminated

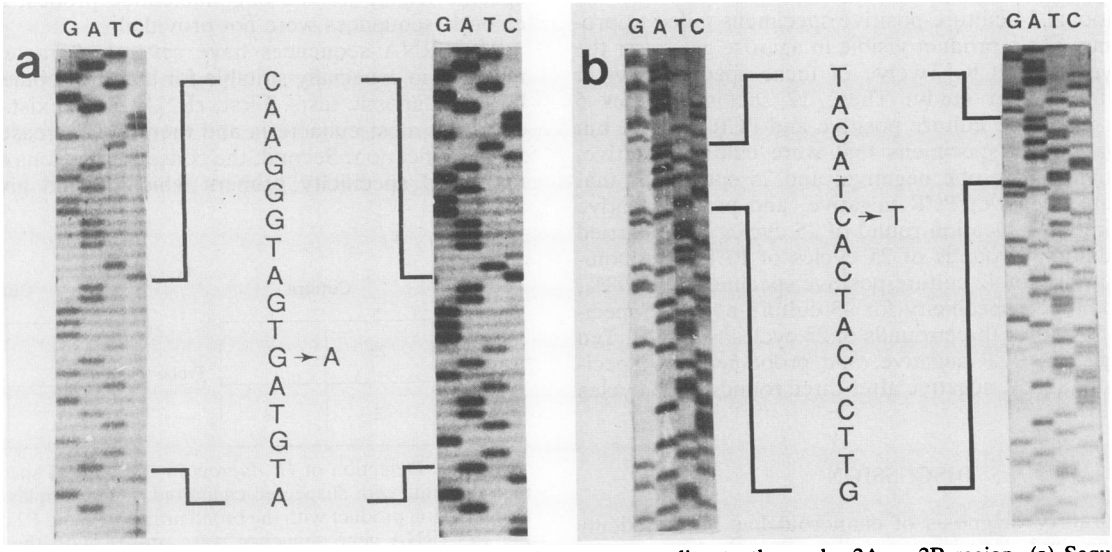


FIG. 3. Autoradiogram showing the sequence of the PCR product corresponding to the probe 2A or 2B region. (a) Sequence of the single-stranded PCR product with the P1 broad primer. (b) Sequence of the single-stranded product with the P2 broad primer. Sequences on the left were observed with isolates hybridizing to the probe 2A sequence. Sequences on the right were observed with isolates hybridizing to the probe 2B sequence.

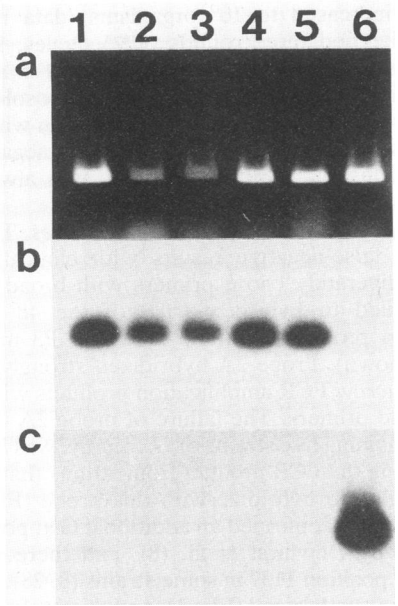


FIG. 4. Sequence specificity of the probe 2A and 2B oligonucleotides. (a) Ethidium bromide-stained agarose gel showing the PCR products generated with the P1 broad and P2 broad primers for six representative strains of *H. ducreyi*. Lanes: 1, strain 557; 2, strain 10945; 3, strain C147; 4, strain SA3114; 5, strain 9468; 6, strain V1157 (representative of the 46 strains hybridizing with probe 2A). (b) Autoradiogram developed after Southern transfer of the PCR products from panel a and hybridization with probe 2B. (c) Autoradiogram developed after Southern transfer of the PCR products from panel a and hybridization with probe 2A.

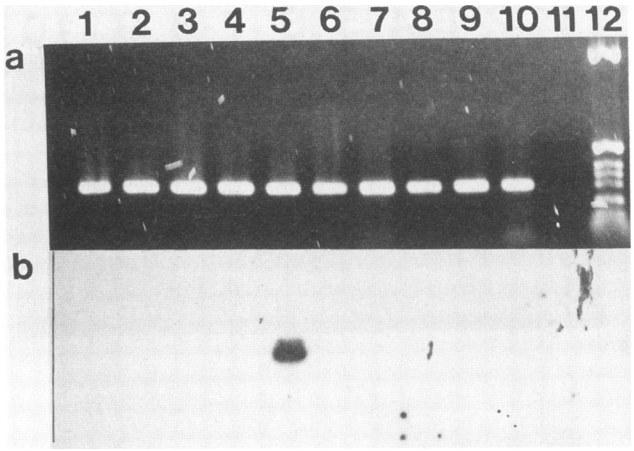


FIG. 5. Sensitivity of the P1 broad and P2 broad primers and specificity of probe 2A. (a) Ethidium bromide-stained agarose gel showing the PCR products generated with the P1 broad and P2 broad primers for 10 species of eubacteria. Lanes: 1, *P. ureae*; 2, *H. haemolyticus*; 3, *A. pleuropneumoniae*; 4, *A. equuli*; 5, *H. ducreyi* V1157; 6, *Haemophilus* sp. (minor group 202); 7, *K. pneumoniae*; 8, *S. typhimurium*; 9, *E. coli*; 10, *P. aeruginosa*. Lane 11 is a negative control containing PCR reagents only. Lane 12 contains DNA size markers as in Fig. 2. (b) Autoradiogram developed after transfer of the PCR products and hybridization with probe 2A.

upon culture, and the status for *H. ducreyi* could not be determined. Fifty-seven of the remaining specimens produced an amplifiable product visible in agarose gels by using the P1 broad and P2 broad primers after the first 25 cycles of PCR (Fig. 6a). The sensitivity for 30 culture-positive specimens was 83%, and the specificity for 27 culture-negative specimens was 67%.

An additional 14 culture-positive specimens failed to produce an amplifiable product visible in agarose gels after the first 25 cycles of PCR. Twelve of these specimens were available for further study. These 12 specimens plus 5 specimens that were culture positive and PCR positive but probe negative; 13 specimens that were culture negative, PCR positive, and probe negative; and 25 specimens that were culture negative, PCR negative, and probe negative after the first amplification round of 25 cycles were carried through additional rounds of 25 cycles of PCR. The combined sensitivity for 42 culture-positive specimens was 98%, and the combined specificity for 43 culture-negative specimens was 51% after three rounds of 25 cycles (Fig. 6b). Ten culture-negative, PCR-negative, and probe-negative specimens remained PCR negative after three rounds of 25 cycles of PCR.

DISCUSSION

The laboratory diagnosis of chancroid has been difficult since the original description of the etiological agent, *H. ducreyi*, by Auguste Ducrey over 100 years ago (1, 21). The absence of effective serological tests (22, 30), the difficulties in culture diagnosis (9, 24, 31), and the increased incidence of disease in North America in recent years (31) have heightened the need for nonculture diagnostic tests.

DNA diagnostic tests are particularly attractive because *H. ducreyi* appears to belong to a monospecific genus only distantly related to other members of the family *Pasteurellaceae* (8). In addition, the current availability of amplification procedures will significantly improve the sensitivity of DNA-based diagnostic tests (17).

Specific probes for the identification of *H. ducreyi* have been readily developed (26) because there is very little homology between strains of *H. ducreyi* and other eubacteria. This has been exploited by Rossau et al. (28) to develop specific rRNA-derived oligonucleotide probes, but the nucleotide sequences were not provided.

The rRNA sequences have several characteristics that make them especially suitable for the development of PCR-based diagnostic tests. First, rRNA genes exist in multiple copies in most eubacteria and therefore increase the signal for amplification. Second, the conserved regions can be used for broad specificity primers which should give positive

a

Probe	Culture +	Culture −
+	25	9
−	5	18

b

Probe	Culture +	Culture −
+	41	21
−	1	22

FIG. 6. Detection of *H. ducreyi* in 100 clinical specimens from male patients with suspected chancroid. Only samples with a PCR amplification product with the broad primers P1 and P2 derived from the 16S rRNA gene sequence were probed with the *H. ducreyi*-specific probe 2A or 2B and included in the tables. Three specimens were contaminated, and culture status could not be determined. Data after one (a) and three (b) rounds of 25 cycles of PCR are shown.

signals with virtually all specimens, providing internal controls for the amplification reaction with clinical material. Finally, the variable regions can be used for the development of species-specific probes. The genetic homogeneity of *H. ducreyi* strains should allow the development of tests with high specificity without loss of sensitivity.

We evaluated several primers and probes for the development of the PCR for the diagnosis of chancroid. The P1 broad and P2 broad primers amplified all bacterial species tested and produced a 303-bp product from clinical specimens. PCR amplification with laboratory isolates was 100% sensitive and specific when used with a pair of 16-base oligonucleotide probes (probes 2A and 2B). Preliminary utilization of these primers and probes to detect *H. ducreyi* in ulcer material from a highly endemic area showed these sequences to be 83 to 98% sensitive and 51 to 67% specific, depending on the number of PCR cycles used for amplification, compared with the culture isolation of *H. ducreyi*. Because the culture sensitivity in the Special Treatment Clinic in Nairobi has been reported to be only about 85% at best, it is entirely likely that the lower specificity is due to false-negative cultures rather than false-positive PCR tests. Using the primers, probes, and methods described, we would recommend probing only samples with a PCR amplification product visible in agarose gels, and we would not amplify beyond three rounds of 25 cycles. Eighty-five of the 95 specimens (89%) processed in the Special Treatment Clinic in Nairobi and transported to Edmonton were evaluable when these criteria were used.

Improvements in the methods by carefully evaluating transport media and conditions, as well as the development of nonradioactive liquid-phase hybridization reactions and amplification methods other than PCR, should facilitate the ease of diagnosis of chancroid and allow laboratory confirmation of the clinical diagnosis of chancroid in remote areas.

Finally, it is possible that multiple agents may be present in genital ulcers. The presence of *H. ducreyi* detected by PCR cannot exclude syphilis, herpes simplex virus, or other organisms associated with genital ulcers. Specific probes for the 16S rRNA sequences of other eubacteria could be utilized with the primers described for amplification here, but agents other than eubacteria would require the development of additional amplification primers and probes.

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