The *rrs* (16S)–*rrl* (23S) ribosomal intergenic spacer region as a target for the detection of *Haemophilus ducreyi* by a heminested-PCR assay

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The intergenic spacer region between the *rrs* and *rrl* ribosomal RNA genes of *Haemophilus ducreyi* was analysed and the DNA sequence was used for the selection of specific PCR primers. A highly sensitive and specific heminested-PCR assay for the identification of *H. ducreyi* was developed. The assay showed a sensitivity of 96% on genital ulcer specimens from patients with clinically diagnosed chancroid, compared with a sensitivity of 56% for culture methods. These results indicate that this PCR assay has the potential to become an accurate and easy reference method for the detection of *H. ducreyi*.

Keywords: *rrs*–*rrl* spacer, *Haemophilus ducreyi*, chancroid

INTRODUCTION

Haemophilus ducreyi, the causative agent of chancroid, is a major cause of genital ulcer disease (GUD) in developing countries. In Africa, Latin America and Southeast Asia, chancroid may account for 10 to 40% of sexually transmitted disease (STD) clinic visits (Kreiss *et al.*, 1989; Morse, 1989; Ortiz-Zepeda *et al.*, 1994). Especially in South Africa, chancroid is a leading infection in men with GUD, with *H. ducreyi* being isolated from 40–68·3% of persons with STDs (Pham-Kanter *et al.*, 1996). GUDs are independent risk factors for human immunodeficiency virus (HIV) infection (Jessamine *et al.*, 1990; Plummer *et al.*, 1991; Grosskurth *et al.*, 1995). GUDs include syphilis, genital herpes, chancroid, lymphogranuloma venereum, and donovanosis. A diagnosis based solely on clinical presentation has an accuracy of 22–80% because of atypia, mimicry and coinfection (Choudhary *et al.*, 1982; Fast *et al.*, 1984; Taylor *et al.*, 1984; Dangor *et al.*, 1990, 1993; Trees & Morse, 1995). A definite diagnosis of chancroid is based on isolation of *H. ducreyi*, a fastidious organism requiring complex media and specific conditions for

growth, making culture difficult and time-consuming and resulting in a sensitivity that varies from 69 to 84% in experienced laboratories (Dangor *et al.*, 1990, 1993; Trees & Morse, 1995).

Non-culture methods using polyclonal and monoclonal antibodies for antigen detection, with immunofluorescence or enzyme immunoassays, have been described by several groups and compared with clinical diagnosis, culture, or nucleic-acid-based detection systems. The accuracy of these tests is not well known (Karim *et al.*, 1989; Roggen *et al.*, 1993; Ahmed *et al.*, 1995). The detection limit of 10⁴ c.f.u. observed with monoclonal antibodies and with DNA probes is a major drawback for the detection of *H. ducreyi* in clinical specimens. Also, inter-study comparisons of test sensitivities are difficult when the denominator is 'the number of patients with chancroid', since the validity of that number depends on the accuracy of the clinical diagnosis. PCR offers a more sensitive approach for detecting low numbers of organisms in clinical specimens. Several PCR assays targeting different DNA sequences have been described for the diagnosis of chancroid (Chui *et al.*, 1993; Johnson *et al.*, 1994, 1995; Parsons *et al.*, 1995; West *et al.*, 1995; Orle *et al.*, 1996). A highly sensitive, easy and very specific diagnostic approach may be the use of the *rrs*–*rrl* intergenic spacer region (ISR) as a target for DNA amplification to identify pathogenic bacteria at the genus or species level (Barry *et al.*, 1991; Jensen *et al.*, 1993).

Abbreviations: ISR, intergenic spacer region; GUD, genital ulcer disease; STD, sexually transmitted disease.

The GenBank accession numbers for the nucleotide sequences reported in this paper are Y11738 for HD CIP 542^T and Y11717 for HD ITM 5600.

Table 1. Micro-organisms used in this study

Micro-organism	Origin	n	Reference*
<i>Haemophilus ducreyi</i>			
Reference strains			
	Vietnam	1	CIP 542 ^T
	USA	4	V 1168, 1169; CF 103, 104
	Sweden	5	EF 7311, 7781, 7829, 9276, 10045
Clinical isolates			
	Zaire	9	ITM 5565 to ITM 5573
	South Africa	7	ITM 5594 to ITM 5600
	Senegal	4	ITM 5629; ITM 5631 to ITM 5633
	Rwanda	9	ITM 5701, 5702, 5704, 5708, 5710, 5716; ITM 5718 to ITM 5720
	Ivory Coast	9	ITM 5800 to ITM 5808
	The Gambia	6	ITM 5604 to ITM 5609
	The Netherlands	2	ITM 3125, 3130
	Thailand	4	ITM 3515; ITM 3519 to 3521
	France	3	ITM 3294 to ITM 3296
	Mexico	1	ITM 3533
	Kenya	5	ITM 2619, 2683, 2684, 2695, 2696
Non-<i>Haemophilus ducreyi</i>			
<i>Neisseria gonorrhoeae</i>		2	ITM 92-502989, 92-502990
<i>Haemophilus influenzae</i>		4	ITM 4772, 4473, 4475, 5252
<i>Haemophilus aegyptius</i>		1	ITM 859
<i>Haemophilus aphrophilus</i>		1	ITM 5323
<i>Haemophilus haemolyticus</i>		1	ITM 1451
<i>Haemophilus parahaemolyticus</i>		1	ITM 999
<i>Haemophilus parainfluenzae</i>		1	ITM 1437
<i>Haemophilus somnus</i>		1	ITM 3610
<i>Haemophilus equigenitalis</i>		1	ITM 3611
<i>Taylorella equigenitalis</i>		1	ITM 2068
<i>Pasteurella gallinarum</i>		1	ITM 1937
<i>Pasteurella haemolytica</i>		1	ITM 1639
<i>Pasteurella multocida</i>		1	ITM 4927
<i>Pasteurella pneumotropica</i>		1	ITM 1299
<i>Pasteurella multocida</i> subsp. <i>septica</i>		1	ITM 1065
<i>Pasteurella ureae</i>		1	ITM 243
<i>Yersinia pseudotuberculosis</i>		1	ITM 34
<i>Actinobacillus hominis</i>		1	ITM 3167
<i>Actinobacillus</i> <i>actinomycetemcomitans</i>		1	ITM 1276
<i>Gardnerella vaginalis</i>		1	ITM 3334
<i>Escherichia coli</i>		1	ITM 1695
<i>Staphylococcus aureus</i>		1	ITM 8
<i>Bacillus subtilis</i>		1	ITM 37
<i>Streptococcus faecalis</i>		1	ITM 5458
<i>Streptococcus faecium</i>		1	ITM 5052
<i>Lactobacillus acidophilus</i>		1	ITM 1033
<i>Candida albicans</i>		1	ITM C31
<i>Treponema phagedenis</i>		1	ATCC 27087
<i>Chlamydia trachomatis</i> LGV2		1	ITM X33
Herpes simplex virus type II		1	ITM X34

* CIP, Collection de l'Institut Pasteur, Paris, France; ITM, Institute of Tropical Medicine, Antwerp, Belgium; EF, collection of the University of Göteborg, Department of Microbiology, University of Göteborg, Sweden; V and CF, collection of the University of Washington, Department of Microbiology, University of Washington, Seattle, USA; ATCC, American Type Culture Collection, Rockville, MD, USA; T, type strain.

In this study we have investigated and characterized the *rrs-rrl* ISR from the type strain of *H. ducreyi*, CIP 542^T and from a recent *H. ducreyi* clinical isolate (ITM 5600). Specific primers were selected to develop a heminested-PCR strategy for the detection of *H. ducreyi* in clinical specimens.

METHODS

Micro-organisms, clinical specimens and culture conditions.

All micro-organisms used in this study (Table 1) were selected from our own culture collection and were cultivated on appropriate media according to standard procedures except *Chlamydia trachomatis* LGV2, Herpes simplex virus type 2 and *Treponema phagedenis* (ATCC 27087) isolated from human syphilis. The genital ulcer specimens were obtained from miners with a clinical diagnosis of chancroid attending an STD clinic at the Leslie William's Memorial Hospital in Carletonville, South Africa. Culture of these samples was performed at the South African Institute for Medical Research as described previously (Dangor *et al.*, 1992). Swabs from the ulcers were stored dry in cryovials at -70 °C and transported to Antwerp on dry ice.

Negative control groups. Swabs from 32 patients with non-genital ulcers caused by different micro-organisms were collected at Benin and Togo in Africa. Genital swabs from 19 patients with non-chancroid STDs and vaginal swabs from 21 healthy females were collected at the STD clinic of the Institute of Tropical Medicine in Belgium.

DNA Extraction. Cultured cells were lysed by heating at 95 °C in 0.05 M NaOH for 10 min. After cooling to room temperature, 0.1 vol 1 M Tris/HCl (pH 8.0) was added. For the clinical samples, 0.5 ml MilliQ water was added to the swabs and the vials were shaken for 30 min at 1000 r.p.m. The swabs were then squeezed and removed. A volume of 95 µl was lysed by adding 5 µl 1 M NaOH and heating at 95 °C for 10 min. After cooling, 10 µl 1 M Tris/HCl (pH 8.0) was added. For amplification, 5 µl lysed specimen was added to 20 µl PCR mixture.

Amplification and direct sequencing of the *rrs-rrl* ISR. The primers used to amplify the spacer region were selected from conserved sequences in the *rrs* and *rrl* rRNA genes (Rossau *et al.*, 1991). Primer U1 was selected at the 3' end of *rrs* and primer R1 corresponded to position 60–44 of the *rrl* sequence (Table 2). Bacterial DNA (50 ng) was amplified using 1 µM of both primers in a 50 µl PCR mixture containing 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM each of the four deoxynucleotide triphosphates, and 2.5 U DNA

polymerase (AmpliTaq, Perkin Elmer). After a first denaturation at 95 °C for 1 min, the reactions were cycled to 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for a total of 30 cycles with a final extension step at 72 °C for 7 min, using a GeneAmp 9600 thermal cycler (Perkin Elmer). After purification on spin columns (Qiaquick PCR purification kit, Qiagen), the PCR products were sequenced using the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems) and analysed on an ABI 373 DNA sequencer.

Amplification and sequencing after cloning. The primers used were U1 (described above), and R2 corresponding to position 478–458 of the *rrl* gene (Table 2). *H. ducreyi* bacterial DNA (50 ng) was amplified using 0.8 µM of both primers in a final volume of 25 µl PCR mixture (compare with above) and 1 U AmpliTaq DNA polymerase. The PCR conditions were as follows: a denaturation step at 94 °C for 3 min; 32 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; followed by a final extension step at 72 °C for 7 min. Amplified products were cloned using the TA cloning kit (Invitrogen). Full-length clones were detected by a rapid alkaline lysis method, restriction analysis, and agarose gel electrophoresis as described by Sambrook *et al.* (1989). Plasmid DNA was purified using the plasmid mini-kit (Qiagen). Both strands of the plasmid insert DNA were sequenced with the M13 universal and reverse primers (Table 2) using the Sequenase fluorescent labelled primer cycle sequencing kit (Amersham). The products were analysed on an ALF DNA sequencer (Pharmacia).

ISR heminested PCR. For diagnostic purposes, a one-tube heminested PCR was developed to amplify part of the *rrs-rrl* ISR using two outer primers (PS1 and PS3) and one inner primer (PS2) (Table 2). The sequence of primers PS1 and PS3 correspond to positions 50–68 and 326–309 of the ISR, respectively (Fig. 1). The sequence of primer PS2 corresponds to position 246–223 of the ISR (Table 2). Either 50 ng bacterial DNA or 5 µl lysed clinical specimens was amplified using 0.8 µM PS1, 0.2 µM PS3 and 0.6 µM PS2 in a final volume of 25 µl. PCRs contained 16.6 mM (NH₄)₂SO₄, 67 mM Tris/HCl (pH 8.8), 10 mM β-mercaptoethanol, 6.7 µM EDTA, 17 µg BSA ml⁻¹, 5% (v/v) DMSO, 1.5 mM MgCl₂, 200 µM each deoxynucleotide triphosphate, and 1 U AmpliTaq DNA polymerase. After an initial denaturation step of 94 °C for 3 min, the reactions were cycled 10 times to 94 °C, 55 °C and 60 °C for 1 min at each step, followed by 35 cycles at 94 °C, 50 °C and 60 °C for 1 min at each step, with a final extension at 72 °C for 7 min.

rrs PCR. For comparison and confirmation, the one-tube nested *H. ducreyi* *rrs* PCR described by West *et al.* (1995) was

Table 2. Sequence of primers used for PCR amplification and sequencing

Name	Sequence (5'–3')	Target (position)
U1	TGGGGTGAAGTCGTAACAAGGTA	<i>rrs</i> (1441–1463)
R1	CACGTCCTTCATCGCCT	<i>rrl</i> (60–44)
R2	CCTTTCCCTCACGGTACTGGT	<i>rrl</i> (478–458)
M13 universal	GTAACACGACGGCCAGT	M13 cloning vector
M13 reverse	TTCACAGGAAACAG	M13 cloning vector
PS1	GACAAGTCGGAATACATCT	ISR (50–68)
PS2	GATTACTCAGACTTTCTACTTTAG	ISR (246–223)
PS3	TATGCGCGAGGCATATTG	ISR (326–309)

slightly modified. We used 0.4 nM of outer primers HD996 and HD1400, and 480 nM of inner primers HD1078 and HD1279 in a final volume of 25 µl. The amplification reaction was performed in two consecutive phases of 10 and 37 cycles.

Agarose gel electrophoresis. PCR products were separated by agarose gel electrophoresis on 2% (w/v) agarose gels, and visualized by UV transillumination after ethidium bromide staining.

RESULTS

Sequence analysis of the *rrs-rrl* ISR

The sequence of the *rrs-rrl* ISR from the type strain of *H. ducreyi*, CIP 542^T, was determined by direct cycle sequencing. For *H. ducreyi* isolate ITM 5600, sequencing of PCR products and five individual recombinant plasmids was performed. All sequences obtained for ITM 5600 were identical, and differed from the CIP 542^T sequence by a single nucleotide, at position 328 of the sequence (Fig. 1). The *rrs-rrl* ISR of *H. ducreyi* consisted of 347 bp containing a single tRNA^{Glu} gene (76 bp). The G + C content was 37.2 mol% and 37.5 mol% for CIP 542^T and ITM 5600, respectively.

Heminested ISR and *rrs* PCR

A fragment of 277 bp was amplified with the outer primers PS1 and PS3, and an internal fragment of 197 bp was amplified with primers PS1 and PS2 (Fig. 2). As estimated by the agarose gel band intensity of PCR products originating from *H. ducreyi* genomic DNA diluted from 50 ng to 5 fg (Fig. 3), the detection limit of this heminested PCR was 50 fg input DNA, which, according to the genome size of *H. influenzae* (Fleischmann *et al.*, 1995), accounts for approximately 25 genome-equivalents.

The sensitivity and specificity of the ISR heminested PCR were determined using 69 strains and clinical isolates of *H. ducreyi*, and 34 non-*H. ducreyi* reference strains selected among species belonging to genera genetically related to *Haemophilus* (such as *Actinobacillus* and *Pasteurella*) and species commonly isolated from the genital tract (Table 1). All 69 *H. ducreyi* strains and isolates, originating from 14 different geographical areas, showed the 197 bp amplified fragment and were also positive with the *rrs* PCR. The 34 non-*H. ducreyi* organisms were all negative for both the ISR and *rrs* PCR assays.

Evaluation of the ISR and *rrs* PCR on genital ulcer specimens

Genital ulcer specimens from 28 patients with a clinical diagnosis of chancroid were cultured and analysed using both the *rrs-rrl* ISR and the *rrs* PCR assays. Identical results were obtained from both PCR assays. One of the 28 specimens was negative in culture and in both PCRs and was considered as clinically misdiagnosed. Of the remaining 27 specimens, 26 (96%) were detected by both PCRs. One culture-positive sample was negative

for both PCRs. The culture method was positive for 15 cases, indicating a sensitivity of 56%.

Evaluation of the ISR and *rrs* PCR on negative control groups

Swabs from 32 patients with non-genital ulcers caused by different micro-organisms, genital swabs from 19 patients with non-chancroid STDs and vaginal swabs from 21 healthy females were collected as negative control groups. All 72 of these swabs were negative in both PCR assays.

DISCUSSION

In this study, the sequence of the intergenic region between the *rrs* (16S) and *rrl* (23S) genes of *H. ducreyi* was analysed, and used to develop a PCR assay for the detection of *H. ducreyi* in genital ulcer specimens. The one-tube heminested PCR described here offers a simple, highly specific and sensitive test with minimum risk of contamination. Although many studies have successfully used the *rrs* (16S) gene for typing and identification purposes, the gene is conserved throughout the *Pasteurellaceae* family (Dewhirst *et al.*, 1992). We selected the region separating the *rrs* and *rrl* genes since this shows sequence variability and length polymorphism which should make it an ideal target for identifying eubacteria at the species level (Barry *et al.*, 1991; Rossau *et al.*, 1992; Rijpens *et al.*, 1995; Gürtler & Stanisch, 1996).

Sequence analysis of the *H. ducreyi* ISR

We used two independent techniques to sequence the spacer region in an attempt to resolve the problems previously encountered, which were considered to be associated with its secondary structure. We obtained identical sequences from the ITM 5600 isolate by these two techniques. Only one nucleotide difference was observed between the ISR sequence of *H. ducreyi* CIP 542^T, isolated in Vietnam during the early 1950s, and ITM 5600, a clinical isolate from South Africa obtained in 1991. The *H. ducreyi* ITM 5600 ISR nucleotide sequence was compared with sequences in the GenBank, EMBL, DDBJ and PDB databases using the BLAST program (Altschul *et al.*, 1997). The only similarity within the spacer region was to the tRNA^{Glu} sequence. In our PCR assay, we used three primers in a heminested-PCR strategy. Ideally, we needed pairs of primers with different annealing temperatures. The variability in the spacer region observed between *H. ducreyi* and closely related species allowed us to select one primer with a low annealing temperature for the first round of amplification, and two primers with higher annealing temperatures for the second round. Each of the three primers is specific for the detection of *H. ducreyi*, in contrast to a previous study where only one primer was specific and the others could anneal to DNA from many members of the *Pasteurellaceae* family. The results of the heminested PCR performed on *H. ducreyi* and non-*H. ducreyi* strains and isolates confirm that the ISR of *H.*

	10	20	30	40	50	60
CIP 542 ^T	CCAAAATAAGACATCACAAGTACTCACACAGATTGTTTGTATTGTTTACACAAGTCGGA					
ITM 5600						
	70	80	90	100	110	120
CIP 542 ^T	ATACATCTTTAAATGTTGTCCCATCGTCTAGAGGCTAGGACATCGCCCTTTCACGGCG					
ITM 5600						
	130	140	150	160	170	180
CIP 542 ^T	GTAACCGGGGTTTGAATCCCGTGGGGACGCCATCTAAAGATGATTTTATTGTCTTATG					
ITM 5600						
	190	200	210	220	230	240
CIP 542 ^T	TTCTTTAAAAAATAGAAACAAGCTGAAACTGAGAGATTTTCTAAAGTAGAAAGTCTGA					
ITM 5600						
	250	260	270	280	290	300
CIP 542 ^T	GTAATCTAAAATCTTAGCTGAACAAAGCAGCTAAGTGTCTAGTCTAAATCATTAAACCAC					
ITM 5600						
	310	320	330	340		
CIP 542 ^T	AAGTATATCAATATGCCTCGCGCATAATAAAATACTTGAGGTTGTAT [347]					
ITM 5600	C..... [347]					

Fig. 1. Sequence alignment of the *rrs-rrl* ISR of *H. ducreyi* CIP 542^T and *H. ducreyi* isolate ITM 5600. The tRNA^{Glu} sequence is underlined. Primer sequences are printed in bold italics.

Fig. 2. Ethidium bromide stained agarose gel displaying the results of *rrs-rrl* ISR heminested PCR on genital ulcer specimens collected from South Africa. Lanes 1–3 and 5, genital ulcer specimens; lane 4, *H. ducreyi* CIP 542^T; lane 6, *H. influenzae* isolate ITM 4473; lane 7, negative control (no DNA); lane 8, 100 bp DNA ladder.

ducreyi is highly conserved at the species level but differs from that of other micro-organisms, including species belonging to the genera *Haemophilus*, *Pasteurella*, and *Actinobacillus*, genetically related to *H. ducreyi* (Rossau *et al.*, 1991; Dewhirst *et al.*, 1992).

Detection of *H. ducreyi* by PCR

DNA amplification techniques are becoming reference methods for the detection of slow-growing and fastidious pathogenic micro-organisms. Several *H. ducreyi*-specific PCR assays have recently been described. Chui

Fig. 3. Ethidium bromide stained agarose gel displaying the analytical sensitivity of ISR heminested PCR. Lane 1, 100 bp DNA ladder; lanes 2–9, PCR products of DNA diluted from 50 ng to 5 fg by 10-fold dilutions; lane 10, negative control (no DNA).

et al. (1993) used broad primers on the *rrs* gene and two *H. ducreyi*-specific probes internal to this sequence. Depending on the number of cycles of amplification, they obtained sensitivities of 83% and 98% and specificities of 67% and 51% relative to culture. Johnson *et al.* (1994, 1995) developed a PCR assay with two primers selected from the sequences of an anonymous fragment of DNA cloned from *H. ducreyi*. A 1100 bp PCR product was detected by Southern blotting and hybridization to a ³²P-labelled probe consisting of the entire sequence. In a first study (Johnson *et al.*, 1994), they observed a sensitivity of 62% and a specificity of 52% relative to culture techniques. By modifying sample preparation, they found a sensitivity of 100% and a specificity of 84% compared with culturing (Johnson *et al.*, 1995). West *et al.* (1995) described a simplified *rrs*-based one-tube nested PCR. The inner primers were labelled with biotin and digoxigenin, and the PCR products were detected using an ELISA. Compared to clinical diagnosis, this assay

showed a sensitivity of 83% while culture techniques showed a sensitivity of 77%.

The sensitivities and specificities observed in the PCR studies cited above are rough estimates since culturing was usually used in the denominator. The relatively low PCR specificities may reflect the poor sensitivity of the *H. ducreyi* culture in these studies. Evaluation of the accuracy of a PCR assay requires comparison with another DNA amplification method, targeting a different gene. Parsons *et al.* (1995) described a *H. ducreyi* PCR using the *groEL* gene as target, and used a confirmatory PCR as described by Johnson *et al.* (1994). They amplified a 505 bp fragment and detected the products by Southern blotting and hybridization with a ³²P-labelled internal oligonucleotide probe. The estimated sensitivity of the *groEL* PCR, compared with culturing and partially with the confirmatory PCR, was 92%. Orle *et al.* (1996) described a multiplex PCR for the simultaneous amplification of DNA targets from *H. ducreyi*, *Treponema pallidum*, and herpes simplex virus. Biotinylated primers targeting the *rrs* gene of *H. ducreyi* were used to amplify a 440 bp fragment, and colorimetric detection was performed using an internal capture probe. The *groEL* PCR described by Parsons *et al.* (1995) was used for confirmation. After discrepant analysis, the sensitivities were 98% for PCR and 74% for culture techniques.

In this study, a heminested-PCR protocol was designed to enhance the sensitivity of the assay. Three specific PCR primers, targeting a unique sequence of *rrs-rrl* ISR of *H. ducreyi*, were used to avoid non-specific amplification when the number of cycles is greater than 35. The specificity analysis of each primer was performed with the FASTDB program. No 100% homologous sequence, other than the *H. ducreyi* sequence, was found in GenBank. Similarity with other species is only found outside the region of the primers. With this ISR heminested-PCR assay, a sensitivity and a specificity of 100% was obtained using cultured samples. We modified the method described by West *et al.* (1995) for use as a confirmatory test. We found that insufficient *H. ducreyi* specific product was generated to be detected consistently by agarose gel electrophoresis unless the concentration of specific primer was increased 1000-fold over the value given in the published method. Using this modification we observed 100% concordance between the two protocols. Therefore, the use of a second PCR assay targeting a different gene has the potential to become a 'gold standard' method for confirmation of *H. ducreyi* infection.

We used samples from a geographical region with a high relative prevalence of chancroid (Pham-Kanter *et al.*, 1996) and where diagnostic accuracy is reported to be 80% (Dangor *et al.*, 1990). The results of our assay were compatible with clinical diagnosis, with a sensitivity of 93% (26/28) and a specificity of 100% (72/72). Both PCR protocols gave identical results. Of the two discordant samples, the first, which was positive by clinical criteria but negative in both PCR assays and also

by culturing, was considered to be the result of a misdiagnosis. The second sample was positive by culturing, although the isolate was no longer available, so we could not confirm whether our primers could have recognized it. However, the presence of non-specific inhibitors of PCR amplification was excluded, by the amplification of human β -globin gene when human DNA was added to the sample together with the necessary primers. Perhaps our failure to detect *H. ducreyi* in this sample was due to an uneven distribution of bacterial cells in the lesion or an insufficient amount of target DNA on this particular swab. The detection limit of the ISR PCR after gel electrophoresis was approximately 50 fg (Fig. 3), which corresponds to about 25 genome-equivalents. This specimen might have contained a low concentration of DNA which could not be detected on an ethidium bromide stained agarose gel. We expect that detection of the PCR products using other systems may enhance the ISR PCR sensitivity without decreasing its specificity.

Using agarose gel electrophoresis for detection, the ISR PCR assay showed a sensitivity of 96% (26/27) on genital ulcer specimens from patients with clinically diagnosed chancroid, compared to a sensitivity of 56% (15/27) for culture methods. The results indicated that the sequence of the *rrs-rrl* intergenic spacer region can be used as a target for PCR amplification for the specific detection of *H. ducreyi*. The DNA extraction method from dried swabs was rapid and easy to perform. Detection by agarose gel electrophoresis is also cheaper than other methods and can be performed with equipment which is readily available in laboratories of developing countries. A preliminary evaluation on clinical specimens confirmed that a one-tube ISR heminested-PCR protocol is a rapid, reliable and convenient method for confirmatory laboratory diagnosis of chancroid.

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