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ORIGINAL ARTICLE

Development of a heminested polymerase chain reaction assay for the detection of *Haemophilus ducreyi* in clinical specimens

Tsegaye Tekle-Michael PhD, **Eddy Van Dyck** MSc, **Saïd Abdellati** BSc and **Marie Laga** MD PhD

Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium

Summary: Detection of *Haemophilus ducreyi* in genital ulcer specimens by culture lacks sensitivity. To enhance detection, a heminested polymerase chain reaction (PCR) assay was developed targeting the nucleotide sequence of a gene, designated *p*27, which encodes for a 27 kDa *H. ducreyi*-specific protein. The *p27* PCR assay detected all (37/37) *H. ducreyi* strains tested and gave no amplified product from DNA extracts of any of 31 other microorganisms, from 30 non-genital ulcer specimens, or from 29 urethral and vaginal swab specimens collected from non-chancroid STD patients. In genital ulcer disease specimens, compared to combined positive results obtained by culture and a previously described PCR assay, the *p27* PCR assay showed a sensitivity of 91% (48/53). The *p27* PCR assay provides a specific and a sensitive detection of *H. ducreyi* in clinical specimens.

Keywords: *H. ducreyi*, genital ulcers, chancroid, PCR

INTRODUCTION

Haemophilus ducreyi is the aetiologic agent of chancroid, a sexually transmitted disease characterized by painful genital ulcerations. In 50% of cases, the disease progresses to inguinal lymphadenopathy which may be manifested by buboes[1]. Chancroid is prevalent in many developing countries[1–4] and is a major risk factor in the heterosexual transmission of HIV infection in endemic regions[4–6].

In low-resource settings, chancroid is commonly diagnosed on clinical grounds alone. However, the accuracy of the clinical diagnosis can be altered by the prevalence of chancroid in the population, by the experience of the attending clinicians, and by co-infection with syphilis, genital herpes, and lymphogranuloma venereum[3,7,8]. Concomitant infection with HIV can also modify the clinical appearance of chancroid lesions[3].

Culture remains the 'gold standard' method for the detection of *H. ducreyi* in patients with genital ulcer disease (GUD). However, due to the fastidious nutritional requirement and specific growth conditions of the bacterium, isolation rates in experienced centres do not exceed 85%[3,9]. Non-culture techniques such as monoclonal antibody-based immunofluorescence assay, and enzyme immunoassays have been reported, but none of these techniques have gained wide acceptance as diagnostic tools[10–13].

DNA probes derived from sequences encoding *H. ducreyi*-specific proteins have been used for DNA hybridization techniques, and have achieved a sensitivity of 10^4 colony forming units in pure and mixed cultures that consisted of *H. ducreyi*, *Haemophilus influenzae* B, and *Escherichia coli*[14]. However, no published data are available to indicate the diagnostic potential of these probes in clinical specimens. In recent years, PCR assays with different targets on the *H. ducreyi* genome have been described and used to detect *H. ducreyi* in clinical specimens[15–21].

It has been shown that an *H. ducreyi*-specific protein with an apparent molecular mass of 28 kDa was present in all of 63 *H. ducreyi* strains tested by Western immunoblot using a pool of sera of patients with culture-proven chancroid[22]. In this study, the nucleotide sequence generated from a recombinant *E. coli* clone encoding the *H. ducreyi*-specific protein was used to develop a heminested PCR assay for the detection of *H. ducreyi* in clinical specimens.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Haemophilus ducreyi strains collected from diverse geographic origins and bacterial strains closely

Correspondence to: E van Dyck, STD/HIV Research and Intervention Unit, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium
E-mail: evandyck@itg.be

related to *H. ducreyi* or found in the genital tract were used in the development of the PCR (Table 1). *H. ducreyi* and other *Haemophilus* strains were cultured on appropriate media as previously described[22]. *Neisseria gonorrhoeae* was grown on GC agar plates at 36°C in a 5% CO_2 atmosphere. *E. coli* and *Candida albicans* were grown on MacConkey and Sabouraud's agar plates, respectively. *Actinobacillus actinomycetemcomitans, Pasteurella ureae* and other Gram-negative bacteria were grown on 5% sheep blood agar plates and incubated at 36°C. *Chlamydia trachomatis* and herpes simplex virus type 2 culture isolates were received from the Clinical Biology Laboratory at the Institute of Tropical Medicine (ITM), Antwerp, Belgium, and from the Department of Clinical Chemistry, Microbiology and Immunology, Gent, Belgium, respectively.

Clinical specimens and nucleic acid extraction

Genital ulcer disease swab specimens were collected consecutively from 104 male miners with a clinical diagnosis of chancroid attending the STD clinic at the Leslie Williams' Memorial Hospital, in Carletonville, South Africa. The specimens were stored at −70°C and transported on dry ice to ITM, Antwerp, Belgium. Swabs collected simultaneously for *H. ducreyi* culture were processed at the South African Institute for Medical Research (SAIMR) using methods described previously[23]. In addition, 65 clinical specimens collected from GUD patients in Mbeya, Tanzania were received for PCR analysis; *H. ducreyi* culture results for these specimens were not available. Skin ulcer specimens from 30 patients in Benin, West Africa, and 29 urethral

Table 1. Microorganisms used in the study

Organisms	Country of origin	*n*	Collection No.*
Haemophilus ducreyi	Democratic Republic of Congo	5	ITM 4747; ITM 5565 to 5568
	France	3	ITM 3294 to 3296
	Mexico	1	ITM 3533
	The Gambia	5	ITM 5604 to 5608
	The Netherlands	2	ITM 3125, 3130
	South Africa	5	ITM 5594 to 5598
	Senegal	4	ITM 5629; ITM 5631 to 5633
	Sweden	3	ITM 3525, 3527, 3529
	Thailand	4	ITM 3515; ITM 3519 to 3521
	USA	4	ITM 5501, 5502, 5504, 5505
	Vietnam	1	CIP 542 (ITM 2663)
Neisseria gonorrhoeae		2	ATCC 49225, 49226
Haemophilus influenzae		4	ATCC 19418; ITM 953, 3877, 4772
Haemophilus aegyptius		1	ITM 859
Haemophilus aphrophilus		1	ITM 5323
Haemophilus aqui		1	ITM 3611
Haemophilus equigenitalis		1	ITM 2068
Haemophilus haemolyticus		1	ITM 1451
Haemophilus parahaemolyticus		1	ITM 402
Haemophilus parainfluenzae		1	ITM 4972
Haemophilus somnus		1	ITM 3610
Pasteurella gallinarum		1	ITM 1937
Pasteurella haemolytica		1	ITM 1639
Pasteurella multocida		1	ITM 816
Pasteurella pneumotropica		1	ITM 1299
Pasteurella pseudotuberculosis		1	ITM 34
Pasteurella septica		1	ITM 1065
Pasteurella ureae		1	ITM 244
Actinobacillus hominis		1	ITM 3167
Actinobacillus actinomycetemcomitans		1	ITM 1276
Gardnerella vaginalis		1	ITM 3334
Escherichia coli		1	ATCC 25922
Staphylococcus aureus		1	ATCC 25923
Bacillus subtilis		1	ITM 37
Streptococcus faecalis		1	ITM 5458
Streptococcus faecium		1	ITM 5052
Lactobacillus		1	ITM 1033
Candida albicans		1	ITM C31

*ITM=Institute of Tropical Medicine, Antwerp, Belgium; CIP=Collection de l'Institue Pasteur, Paris France; CIP 542, Type strain; ATCC=American Type Culture Collection

and vaginal swab specimens collected from STD patients without GUD from South Africa and Antwerp were used as negative controls.

DNA extraction was performed as described previously[24]. Colonies of bacteria were suspended in distilled water and, in the case of *H. ducreyi*, the suspension was passed through a 26 G needle several times to break the aggregation and then allowed to settle at room temperature for 5 min[25]. The supernatant was then used to prepare a solution to a density equivalent to McFarland tube No. 1 (3×10^8 bacteria/ml). Five μl of 1 N NaOH were added to 95 μl of bacterial suspension and the mixture heated at 95°C for 15 min. After cooling to room temperature, 10 μl of 1 M Tris-HCl (pH 8.0) was added and the suspension was kept at −20°C until use for PCR analysis. The swab specimens from GUD patients and control groups were re-suspended in 0.5 ml MilliQ water by shaking (1000 rpm) at room temperature for 30 min. The swabs were squeezed against the internal wall of the tubes to express the content and then discarded. The suspension was used to extract DNA as described above.

Deletion cloning and DNA sequencing

The presence of *H. ducreyi*-specific protein with an approximate molecular mass of 28 kDa was identified by a Western blot assay in 63 *H. ducreyi* strains[22]. The gene encoding this protein was previously cloned and a recombinant *E. coli* clone (*p*DR935) expressing a 27 kDa was identified. Partially deleted subclones were constructed from *p*DR935, using a double-stranded nested deletion cloning kit according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). Miniprep plasmid DNA isolation was performed using a commercially available kit (Qiagen, Hilden, Germany). Both strands of the template plasmid DNA were sequenced using T7 DNA polymerase and fluorescein labelled M13 universal primers (Pharmacia Biotech). DNA and amino acid sequences were edited and analysed using DNASIS (Hitachi Software Engineering Company, Yokohama, Japan) and Genetic Computer Group (Madison, WI, US) software. The DNA sequence reported in this paper has been deposited in the GenBank under the accession number AF087639.

p27-based heminested PCR

A forward primer, HD51 (5′-AACTGCTTGTGATA AGCCTGC-3′), and 2 reverse primers, HD338 (5′-TTAGCATTTTGTTGGATTTTCTC-3′) and HD454 (5′-GCGTTGGGTTTTTAGCTAATTC-3′), were synthesized by Pharmacia Biotech based on the *p27* nucleotide sequence. PCR reagents were purchased from Perkin-Elmer (Cetus, Norwalk, CT, US), unless otherwise indicated. Amplification reaction mixture, in a final volume of 25 μl, comprised 10 mM Tris-HCl pH 8.3, 2.0 mM $MgCl_2$, 50 mM KCl, 10% dimethyl sulfoxide, deoxyribonucleoside triphosphates mix (200 μM each) (Pharmacia Biotech), 1 μM of each primer, 0.5 U of Taq DNA polymerase and 40 ng of genomic DNA or 4 μl of extract from clinical specimens. PCR was performed by 25 cycles using the HD51/HD454 primer set followed by 35 cycles using HD51/HD338. Amplification conditions were: a denaturation step at 94°C for 3 min followed by cycles of 1 min each at 94°C, at 60°C and at 72°C, respectively, and a final elongation step at 72°C for 7 min in a programmable thermal cycler (Perkin-Elmer). One μl of PCR amplified product from the first round was used as a template for the second round amplification. Parallel positive (containing DNA extracted from type strain *H. ducreyi* CIP 542) and negative (reaction mixture without template DNA) controls were included in each run. The heminested PCR was performed twice on all specimens. To prevent contamination, the preparation and aliquoting of the reaction mixtures and analysis of PCR products were carried out in separate rooms, the working area was UV-irradiated and aerosol-guarded pipette tips were used[26]. PCR amplification products were detected and identified as *H. ducreyi*-specific DNA by visualization of a 288-bp fragment on ethidium bromide-stained agarose gels. The *rrs* (16S)-*rrl* (23S) intergenic spacer region PCR (ISR PCR) assay was used without modification for comparative and confirmatory purposes[21].

p27 PCR assay specificity and sensitivity

To assess the accuracy of the *p27* PCR assay, genomic DNA extracts of *H. ducreyi* strains (n=37) collected from diverse geographic origins and of microorganisms commonly found in the human genital tract or related to *H. ducreyi* (n=31) were used. In addition, DNA extracts of *C. trachomatis* serotypes L1 to L3 and herpes simplex virus type 2 were tested. A series of 10-fold dilutions of genomic DNA from *H. ducreyi* type strain CIP 542 was amplified to determine the sensitivity of the heminested PCR under the amplification conditions specified above. To verify that the DNA extracts did not contain PCR inhibitors, a segment of human β-globin gene was amplified as described previously[27]. The agreement between *p27* PCR and ISR PCR assays was evaluated in GUD specimens from Tanzania (n=65) and a subgroup of specimens from South Africa (n=75) using the kappa statistic[28].

RESULTS

Sequence analysis

Subclones prepared by partial deletion cloning of *p*DR935 were sequenced. An *H. ducreyi* DNA fragment (854 bp) was localized in *p*DR935B37 harbouring a gene (642 bp), designated as *p27* (data not shown). The DNA sequence revealed the presence of an open reading frame with its own

regulatory sequences, encoding for 213 amino acids. A basic local alignment search tool (BLAST) search[29] of the nucleotide sequence comparison with sequences at the GenBank revealed a 94% identity with *hlp*28 gene of *H. ducreyi*, encoding a 28 kDa lipoprotein[29,30].

Sensitivity and specificity of *H. ducreyi* detection

In the heminested PCR amplification protocol, the outer primers HD51/HD454 amplified a 404-bp DNA fragment, while a 288-bp internal fragment was amplified by the HD51/HD338 primer set (Figure 1). The detection sensitivity of the heminested *p27* PCR was determined by amplifying series of 10-fold dilutions of chromosomal DNA extract from *H. ducreyi* CIP 542. The lowest detectable amount of *H. ducreyi* chromosomal DNA was about 0.4 pg (Figure 2).

The suitability of the selected primers for the specific detection of *H. ducreyi* was established using *H. ducreyi* strains (n=37) collected from diverse geographical origins and different periods. The *p27* PCR assay detected all *H. ducreyi* strains tested. In contrast, no amplification product was detected on DNA extracts from *C. trachomatis* serotypes L1 to L3, herpes simplex virus type 2, and 31 microorganisms phylogenetically related to *H. ducreyi* or found in the human genital tract.

Detection of *H. ducreyi* in clinical specimens

The performance of the *p27* PCR for 104 consecutive GUD specimens collected in South Africa is shown in Table 2. Because the comparative ISR PCR was performed on 75 of the 104 GUD specimens, more complete analysis for these specimens is shown in Table 3. The agreement

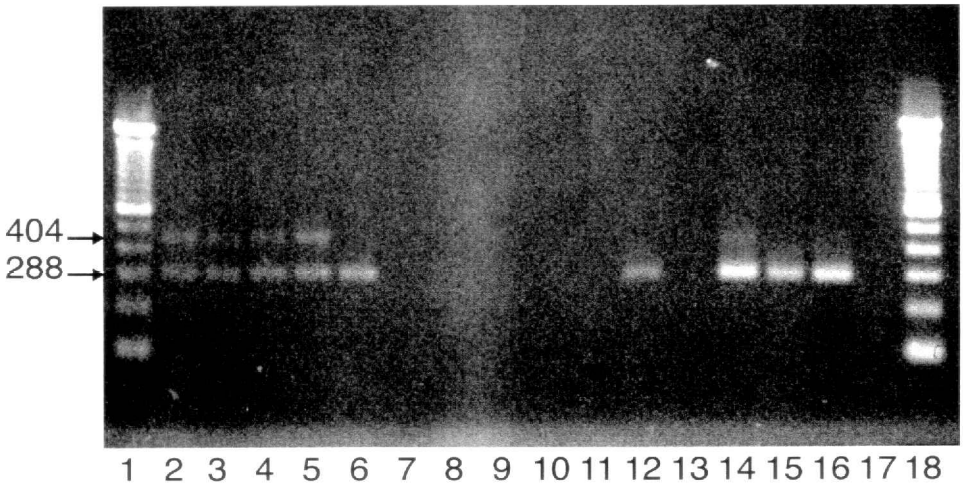

Figure 1. Ethidium bromide-stained agarose gel showing the polymerase chain reaction (PCR) products amplified from H. ducreyi, *non-*H. ducreyi *strains and clinical specimens. Lanes 1,18: 100-bp DNA ladder; 2–6:* H. ducreyi *CIP 542, ITM 4747, 3533, 5566, 3529; 7–11:* N. gonorrhoeae *ATCC 49225,* H. influenzae *ATCC 19418,* H. parainfluenzae *ITM 4972,* P. ureae *ITM 244,* E. coli *ATCC 25922; 12–16: clinical specimens; 17: negative control. Lanes were loaded with second round PCR amplification products*

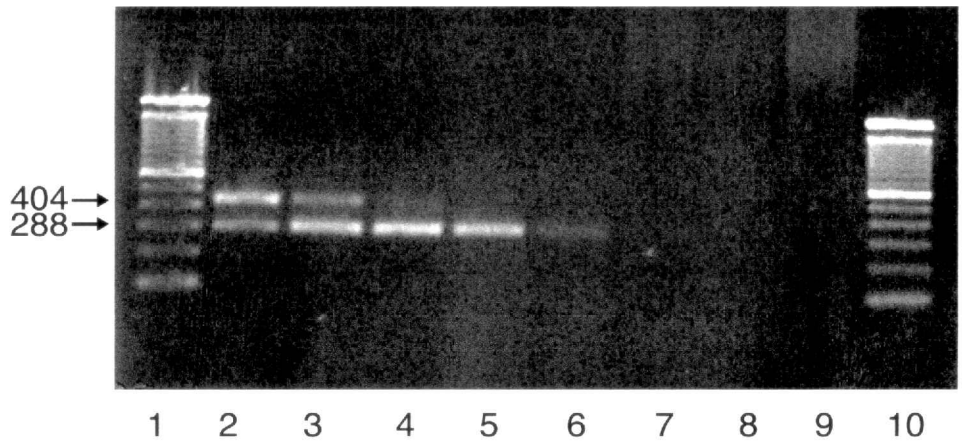

Figure 2. Sensitivity of the heminested p27 PCR. Ethidium bromide-stained agarose gel showing the polymerase chain reaction (PCR) products amplified from a serial 10-fold diluted DNA sample of type strain H. ducreyi *CIP 542. Lanes 1, 10: 100-bp DNA ladder; 2–8: 4 ng – 4 fg DNA; 9: negative control. Lanes were loaded with second round PCR amplification products*

Table 2. Detection of Haemophilus ducreyi *in clinical samples by p27 PCR compared to culture*

	H. ducreyi culture		
p27 PCR	Positive	Negative	Total (%)
Positive	46	22	68 (65.4)
Negative	4	32	36 (34.6)
Total (%)	50 (48.1)	54 (51.9)	104

PCR=polymerase chain reaction

between ISR PCR and *p27* PCR was 92% (kappa score=0.82). For 65 GUD specimens from Tanzania, 11 were ISR PCR and *p27* PCR positive, 53 were ISR PCR and *p27* PCR negative, and one specimen was ISR PCR positive and *p27* PCR negative, resulting in 98.5% agreement between both amplification assays (kappa score=0.95). Compared to ISR PCR, *p27* PCR and culture had sensitivities of 92% (48/52) and 65% (34/52), respectively. If one considers culture and ISR PCR as an expanded 'gold standard', 53 clinical specimens were positive for *H. ducreyi*, with a resolved sensitivity of 91% (48/53) for *p27* PCR assay.

Twenty-nine urethral and vaginal swab specimens collected from non-chancroid STD patients, and 30 non-genital ulcer specimens from patients in Benin, West Africa, were tested alongside the genital ulcer swab specimens: all 59 specimens were negative by the *p27* PCR.

DISCUSSION

Polymerase chain reaction techniques are useful for the direct detection of aetiologic agents which are difficult to grow in artificial media or for which isolation by culture is suboptimal[31]. For the laboratory diagnosis of chancroid, the detection of *H. ducreyi* by culture method is problematic due to lack of sensitivity and PCR assays are increasingly being used as 'reference' methods.

Polymerase chain reaction assays developed for the detection of *H. ducreyi* differ in DNA target sequences and amplification techniques. The methods used to estimate the sensitivity and specificity of these techniques are also different. DNA sequences of an anonymous cloned fragment of *H. ducreyi* was used as a target for a PCR assay[16]. An amplified fragment of 1.1 kb was detected by Southern blotting and hybridization to a ^{32}P labelled 1.1 kb probe[16]. Compared to culture, the PCR assay had a sensitivity of 62% and a specificity of 52%. The low detection rate was attributed to the presence of Taq polymerase inhibitiors in the DNA extract. After altering the specimen preparation protocol, a sensitivity of 100% and a specificity of 84% was reported as compared to culture[17]. The anonymous fragment has recently been designated as *rec*D gene[32]. A nested PCR assay was developed targeting the *rrs* (16S) gene of *H. ducreyi*. Compared to clinical diagnosis, the PCR assay had a sensitivity of 83%[18]. Similarly, the *gro*EL gene of *H. ducreyi* was used as a target for PCR amplification[19]. A 505-bp fragment amplified from 139 *H. ducreyi* isolates was detected by Southern blot using an internal probe labelled with ^{32}P. Using a confirmatory PCR assay, the *gro*EL PCR assay had a resolved sensitivity of 92%; for culture the resolved sensitivity was 77%[19]. A multiplex PCR (M-PCR) assay with colorimetric detection system was developed for the simultaneous detection of *H. ducreyi*, *Treponema pallidum* and herpes simplex virus type 1 and 2[20]. For *H. ducreyi* detection, the *rrs* gene was used as a target to select the primers and a probe. Using the *gro*EL PCR as a confirmatory assay, the resolved sensitivities of M-PCR and culture for *H. ducreyi* detection were 98.4% and 74.2%, respectively[20]. Recently, a heminested ISR PCR assay for *H. ducreyi* has been described[21]. The amplified 197 bp internal fragment was detected by ethidium bromide stained agrose gel. Identical results were obtained in 28 clinical specimens analysed by ISR PCR and a nested *rrs* PCR. Compared to clinical diagnosis, the ISR PCR had a sensitivity of 96%[21].

Table 3. Comparison of p27*-based polymerase chain reaction (PCR) (*p27*),* rrs *(16S)-*rrl *(23S) intergenic spacer region PCR (ISR) and* Haemophilus ducreyi *culture for genital ulcer disease specimen from South Africa*

	H. ducreyi culture		
PCR results	Positive	Negative	Total
p27+/ISR+	30	17	47
p27−/ISR +	3	2	5
p27+/ISR−	0	1	1
p27−/ISR−	1	21	22
Total	34	41	75

In this study, we described a heminested PCR assay targeting the *p27* gene of *H. ducreyi*. Despite the differences in geographical origin and period of collection of the *H. ducreyi* strains, all were detected by the *p27* PCR assay. In contrast, *Haemophilus* spp., *Pasteurella* spp., *Neisseria* spp., and other microorganisms were all negative by the *p27* PCR. The lowest detectable amount of *H. ducreyi* DNA was about 0.4 pg, which roughly corresponds to 10 to 100 organisms[16].

In GUD specimens from 104 mine workers in South Africa, *p27* PCR assay had a sensitivity of 92% (46/50) compared to *H. ducreyi* culture result. This observation is identical to that reported in *H. ducreyi rrs* PCR and similar to that reported in *H. ducreyi gro*EL PCR (89%) assay[18,19]. Compared to a reference combining positive *H. ducreyi* culture and ISR PCR results, the *p27* PCR assay had a resolved sensitivity of 91% which was comparable with 92% resolved sensitivity reported for the *gro*EL PCR[19].

Three culture-positive specimens were negative by *p27* PCR but positive by ISR PCR assay. The

disparity observed could not have been due to PCR inhibition as this was verified by amplifying β-globin gene in human DNA with appropriate primers. It may be attributed to presence of low quantity of target DNA in the swab specimens or the lower DNA detection capacity of *p27* PCR assay compared to the ISR PCR. However, cultured *H. ducreyi* isolates from the specimens with discrepant results were not available to ascertain detection with the primers used in *p27* PCR assay.

The skin ulcer specimens from patients in Africa with advanced, ulcerated active *Mycobacterium ulcerans* infections as confirmed by PCR (F Portaels, personal communication) were intended to verify whether super-infecting or colonizing bacteria interfere in the performance of *p27* PCR assay. All skin ulcer specimens as well as the urethral and vaginal swab specimens from STD patients without GUD were negative.

The nucleotide sequence generated in this study had a high level of identity with a DNA sequence of a gene encoding for *H. ducreyi* lipoprotein, suggesting that the 2 sequences represent the same gene or 2 very closely related genes. Functional analysis of the protein(s) encoded by the gene(s) could provide better insight into the observed nucleotide sequence identity.

The rRNA genes and *gro*EL gene exist in multiple copies in most eubacteria, including *H. ducreyi*[19,33]. Species-specific regions of these evolutionarily conserved genes have been successfully used as targets for PCR assays for the detection and differentiation of pathogenic microorganisms[31]. It is suggested that the high copy number may be useful in increasing sensitivity of detection by PCR. It has yet to be established whether *p27* is a single copy or a multiple copy gene.

In conclusion, the results obtained in this study show that the *p27* PCR assay is specific and sensitive. In addition, the detection method, unlike some of *H. ducreyi* PCR assays, does not involve radio-labelling and hybridization. The DNA extraction protocol was simple and efficient in obtaining sufficient target DNA for amplification from clinical specimens. Thus, the *p27* PCR assay in conjunction with *H. ducreyi* culture or other *H. ducreyi* PCR assays can be used as a 'gold standard' technique to evaluate newly developed laboratory techniques for the diagnosis of chancroid.

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