MAJOR ARTICLE

Detection and Discrimination of Herpes Simplex Viruses, *Haemophilus ducreyi*, *Treponema pallidum*, and *Calymmatobacterium (Klebsiella) granulomatis* from Genital Ulcers

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Background. Genital ulcer disease (GUD) is commonly caused by pathogens for which suitable therapies exist, but clinical and laboratory diagnoses may be problematic. This collaborative project was undertaken to address the need for a rapid, economical, and sensitive approach to the detection and diagnosis of GUD using noninvasive techniques to sample genital ulcers.

Methods. The genital ulcer disease multiplex polymerase chain reaction (GUMP) was developed as an inhouse nucleic acid amplification technique targeting serious causes of GUD, namely, herpes simplex viruses (HSVs), *Haemophilus ducreyi*, *Treponema pallidum*, and *Klebsiella* species. In addition, the GUMP assay included an endogenous internal control. Amplification products from GUMP were detected by enzyme linked amplicon hybridization assay (ELAHA).

Results. GUMP-ELAHA was sensitive and specific in detecting a target microbe in 34.3% of specimens, including 1 detection of HSV-1, three detections of HSV-2, and 18 detections of *T. pallidum*. No *H. ducreyi* has been detected in Australia since 1998, and none was detected here. No *Calymmatobacterium (Klebsiella) granulomatis* was detected in the study, but there were 3 detections during ongoing diagnostic use of GUMP-ELAHA in 2004 and 2005. The presence of *C. granulomatis* was confirmed by restriction enzyme digestion and nucleotide sequencing of the 16S rRNA gene for phylogenetic analysis.

Conclusions. GUMP-ELAHA permitted comprehensive detection of common and rare causes of GUD and incorporated noninvasive sampling techniques. Data obtained by using GUMP-ELAHA will aid specific treatment of GUD and better define the prevalence of each microbe among at-risk populations with a view to the eradication of chancroid and donovanosis in Australia.

Genital ulcer disease (GUD) is commonly caused by pathogens against which suitable therapies exist but for which clinical and laboratory diagnoses may be problematic. The 3 most common etiological agents of genital lesions are the herpes simplex viruses (HSVs), *Treponema pallidum* subspecies *pallidum* (a gram-negative bacterium that causes syphilis), and *Haemophilus ducreyi* (a gram-negative bacteria that causes chancroid). Although chancroid has not been reported in Australia since 1998, the total number of Australian notifications of syphilis have ranged from 1304 notifications in 1997 to 2056 in 2004 [21].

An additional but rare cause of GUD is the gram-negative organism traditionally entitled *Calymmatobacterium granulomatis*. Further characterization of the organism by cell culture [8, 14] and DNA sequencing resulted in the proposed reclassification of the organism to *Klebsiella granulomatis* [5, 9]. The disease has been commonly known as granuloma inguinale, but the preferred name, donovanosis, is less limiting when de-

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scribing the potential of the disease to affect many anatomical sites, resulting in chronic, ulcerative disease [4]. Although it is generally a benign infection, disease can result in extensive tissue destruction and can even be fatal [13]. Donovanosis is endemic in parts of Southern Africa, Brazil, India, Papua New Guinea, the Caribbean, and Central and Northern Australia, including the north of Western Australia [13, 22]. Despite that the number of Australian donovanosis notifications decreased from 117 in 1994 to 16 in 2002 and 2003 [21], clinical underdiagnosis of the condition, in addition to the reluctance of persons to present for treatment, is suspected of falsely depressing prevalence figures [3, 16]. The detection of donovan bodies within large mononuclear cells obtained in smears of lesion or biopsy material remains the conclusive diagnostic approach, requiring careful specimen selection and expert histological investigation [13].

PCR has improved the speed and sensitivity of microbial diagnosis and permitted more-common use of less invasive sampling techniques [6, 23]. Multiplex PCR makes best use of limited amounts of clinical material when multiple targets are under investigation, and it improves cost-effectiveness [27]. *C. (Klebsiella) granulomatis* has not been included in previously reported multiplex PCR assays investigating GUD, despite the existence of a robust PCR technique [7, 10].

We report the first single-tube, multiplex PCR to simultaneously detect 5 microbial agents of GUD together with an endogenous internal control included to determine any effects on amplification caused by inhibitors or degraded template. This collaborative project was undertaken to address the need for a rapid, economical, and sensitive approach to the detection and diagnosis of treatable GUD using noninvasive sampling techniques. The intent of the application of the genital ulcer disease multiplex PCR (GUMP) assay was also to improve understanding of the prevalence of donovanosis in at-risk populations and test the hypothesis that Queensland is chancroid free.

METHODS AND MATERIALS

Clinical specimens. To perform a prospective study of the multiplex PCR, 64 specimens consisting of smear and/or press slides ($n = 3$), swabs ($n = 59$), and tissue biopsies ($n = 2$) were collected. The specimens, as part of routine clinical care, were supplied without detailed clinical histories, but it is known that they were collected from 55 HIV-uninfected subjects (58.9% of whom were male) presenting at remote area health centers in northern Queensland with any acute or chronic genital lesion during 2002. Subjects were not excluded on the basis of previous or ongoing antimicrobial therapy. The subject population predominantly comprised Aborigines and Torres Strait Islanders who ranged in age from 2.6 years to 59.4 years, with a median age of 24.8 years. Results were compared with those of conventional testing, including PCR, bacterial and viral culture, histologic analysis, rapid plasmin reagin (RPR) and Venereal Disease Research Laboratory assays, *T. pallidum* particle agglutination (TPPA), and fluorescent treponemal antibody absorption. Nucleic acids were purified from 200 μL of each resuspended specimen using the High Pure PCR Template Preparation Kit (Roche Diagnostics).

PCR. Multiplex PCR combined 5 μL of synthetic control or purified specimen with 45 μL of PCR mix (4 mM of $MgCl_2$; 50 mM of KCl; 10 mM of Tris-HCl [pH, 8.3]; 0.2 mM of dATP, dCTP, and dGTP; 0.4 mM of dUTP; and 3 μM of digoxigenin-11-dUTP [Boehringer Mannheim]) containing 2 pmol of each oligonucleotide primer (Sigma-Genosys) (table 1) and 2 units of PlatinumTaq DNA polymerase (Invitrogen). The primers for HSV, *H. ducreyi* and *T. pallidum* were chosen from a previous study [23], whereas the primers for *C. granulomatis* were unique to this study. Amplification commenced with a 2-min incubation at 95°C followed by 45 cycles of 20 s at 94°C, 20 s at 62°C, and 30 s at 72°C and a final 7-min incubation at 72°C.

Uniplex PCR containing template DNA was performed as described for multiplex PCR, except only a single primer pair corresponding to 1 target sequence was included. Confirmatory PCR assays were employed for discrepant results for HSV and *T. pallidum* by targeting the polymerase and 16S rRNA genes, respectively [18, 26].

Amplicon was detected by agarose gel electrophoresis or by enzyme linked amplicon hybridization assay (ELAHA), a microwell DNA hybridization method using an enzyme-mediated color reaction to indicate the presence of specific template amplification [19].

Construction of synthetic controls. Positive controls containing cloned templates were assembled for each target, as described elsewhere [19]. Templates were obtained from clinical material. Each primer pair's amplicon was electrophoresed, purified (QIAquick gel extraction kit; Qiagen), and ligated into the pGEM-T Easy Vector System I (Promega). Plasmids containing the insert were transformed into *Escherichia coli* DH5α and expanded in culture, and 1 μL of the transformed bacteria was directly screened by PCR-ELAHA. Positive clones were purified using a mini-prep purification kit (GenElute Plasmid Miniprep Kit; Sigma). The controls were designated pWC*tpal (T. pallidum* subspecies *pallidum)*, pWC*cg (C. granulomatis)*, pWC*hsv1,* pWC*hsv2,* pWC*hd (H. ducreyi)*, and pWC*erv* (endogenous retrovirus 3).

Synthetic control stocks were initially quantified at 260 nm using a UV visible spectrometer (Cintra 20; GBC) then titrated using 10-fold dilutions from 10^7 to 10^0 copies/μL. Each dilution was tested by uniplex PCR to confirm the expected copy number, as described elsewhere [18]. Quantified stock solutions containing adjusted copy numbers in the range of 1×10^9 to 5×10^9 amplifiable targets were stored in 2-μL single-use aliquots at -70°C.

Table 1. Nucleotide sequences and origin of the sense and antisense primers and biotinylated oligoprobes, together with the expected amplicon length.

Oligonucleotide	Oligonucleotide sequence (5′– 3′)	Target gene	Amplicon length (base pair)	Reference
HSV01.16[a]	GCCGTAAAACGGGGACATGTACACAAAGT	Glycoprotein B	432	[23]
HSV02.16	TTCAAGGCCACCATGTACTACAAAGACGT			
HSV1_P01.16[2]	GCGTTGGCCGGTTTCAGCTCC	…	…	This study
HSV2_P02.16	GACCTTCGCCGGCTTGAGCTC			
HD01.1	CAAGTCGAACGGTAGCACGAAG	HD 16S rRNA	439	[23]
HD02.1	TTCTGTGACTAACGTCAATCAATTTTG			
HD_P02.1	CCGAAGGTCCCACCCTTTAATCCGA			
TPAL01.3	CAGAGCCATCAGCCCTTTTCA	47kDa lipoprotein	260	[23]
TPAL02.3	GAAGTTTGTCCCAGTTGCGGTT			
TPAL_P01.3	CGGGCTCTCCATGCTGCTTACCTTA			
ERV01.1	CATGGGAAGCAAGGGAACTAATG	Envelope	136	[29]
ERV02.1	CCCCAGCGAGCAATACAGAATT			
ERV_P01.3	TCTTCCCTCGAACCTGCACCATCAAGTCA			
CG01.1	TCCTCTGCCCAGACCGATAACTTTATG	*phoE*	328	This study
CG02.1	CCAGGTAGATATTGTTGGCGTCA			
CG_P01.1	GCCGTCAGCGCAGCCTACACCAGC			
16SrRNA01.1	CCTAACACATGCAAGTCGA	Bacterial 16S rRNA	556	[9]
16SrRNA01.2	CGGTCTGTCAAGTCGGAT			
16SrRNA02.1	ATCCGACTTGACAGACCG	…	653	…
16SrRNA02.2	TGTAGCACGTGTGTAGCC			

NOTE. CG, *Calymmatobacterium granulomatis;* ERV, endogenous retrovirus 3; HD, *Haemophilus ducreyi;* HSV, herpes simplex virus; TPAL, *Treponema pallidum.*

[1] Forward (01.X) and reverse (02.X) primers.

[2] Oligonucleotide probe is identified by the "P" preceding the 3 digit identification number.

C. (Klebsiella) granulomatis *confirmation.* Confirmation of *C. granulomatis* among *Klebsiella*-positive specimens was performed by digesting the amplicon with 5 units of *Hae*III (Promega) in a total volume of 20 μL (figure 1).

Further confirmation of the presence of *C. granulomatis* required amplification of the 16S rRNA gene using primers 16S rRNA 01.1 and 02.1 (table 1) [9]. Both strands of amplicon representing ~500 bases proximal to the 5′ end of the gene were sequenced (ABI PRISM BigDye v3.1; Perkin Elmer Applied Biosystems Division).

RESULTS

Analytical sensitivity and assay specificity. ELAHA increased the limit of amplicon detection by at least 8-fold, compared with agarose gel electrophoresis of an equal amount of amplicon (data not shown) [18]. GUMP-ELAHA was capable of detecting 3×10^0 copies of each synthetic control template per 50-μL reaction, or, when all 6 templates, including the internal control, were coamplified, GUMP-ELAHA was capable of detecting 3×10^1 copies per reaction (table 2). In addition, all coamplified template loads above the limit of detection could be visualized using agarose gel electrophoresis and discriminated by ELAHA (data not shown).

A wide range of related and unrelated nucleic acid templates were investigated by GUMP-ELAHA, including *Bacillus laterosporus, Bartonella henselae, Citrobacter freundii, Citrobacter koseri, Cryptococcus neoformans, Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella oxzanae, Klebsiella planticola, Enterobacter aerogenes, Enterobacter agglomerans, Enterobacter cloacae, E. coli, Escherichia fergunosii, Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Salmonella* species, *Streptococcus* species, *Serratia* species, *Saccharomyces cerevisiae, Candida* species, *Vibrio alginolyticus, Flavobacterium multivoram, Shigella flexneri, Proteus* species, *Staphylococcus* species, *Neisseria meningitidis, Acinetobacter haemolyticus, Bacillus coagulans,* and human genomic DNA (Promega). The only GUMP primer pair to crossreact with an unintended template was that for the detection of *C. granulomatis,* which amplified some enterobacterial non-GUMP targets.

The GUMP study. A GUMP target was identified in 22 specimens (34.3%), comprising 4 HSV specimens (6.2%) and 18 *T. pallidum* specimens (28.1%), whereas 42 (65.6%) of the 64 specimens remained negative for a target by GUMP-ELAHA (table 3). Twenty specimens (31.2%) were negative for a target by both GUMP-ELAHA and traditional testing. There were 4 instances in which HSV-2 was detected by culture but not by

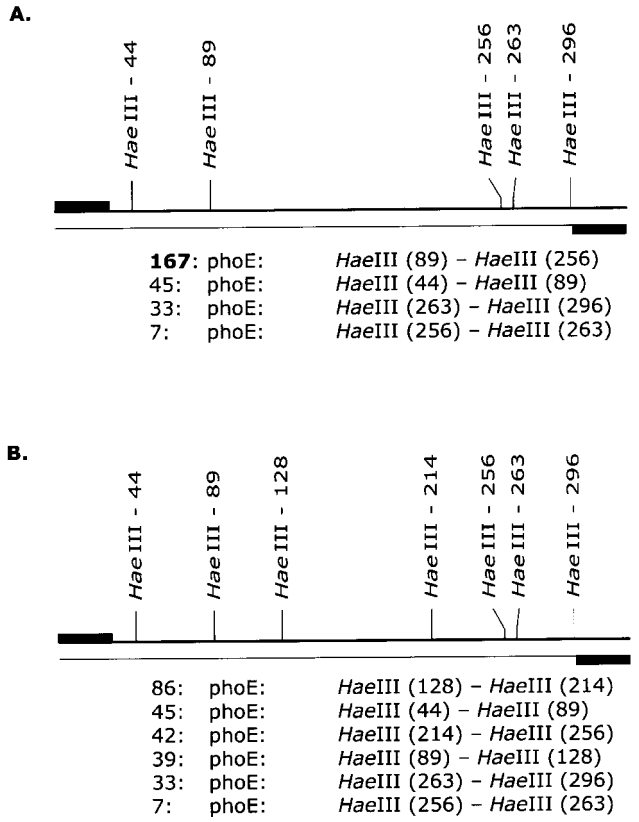

Figure 1. The expected restriction enzyme patterns resulting from *Hae*III restriction of the 328–base pair (bp) phosphate porin *(phoE)* amplicon generated by amplification of *Klebsiella* templates using genital ulcer disease multiplex polymerase chain reaction (primer sites are boxed). The 167-bp fragment *(A)* was diagnostic for *Calymmatobacterium granulomatis* and was absent when unintended bacterial species were amplified and restricted. *B,* The *Klebsiella rhinoscleromatis* sequence is presented as an example of a nontarget species of *Klebsiella*.

GUMP-ELAHA: in 1 instance, HSV-2 was detected by the confirmatory assay; in another instance, detection was affected by amplification failure; and in the remaining 2 instances, the specimens originated from the same subject and repeatedly tested negative by both HSV assays. To further examine the performance of the HSV component of GUMP-ELAHA, 103 previously determined HSV-positive and HSV-negative swab specimens (50 were HSV negative, 24 were HSV-1 positive, 26 were HSV-2 positive, and 3 were dual HSV positive) were examined, and the results demonstrated that the HSV component of GUMP-ELAHA was capable of an analytical sensitivity of 98.2% and a specificity of 96.1%. No subjects were found to be positive for either *H. ducreyi* or *C. granulomatis.* Of the 11 subjects who were negative for *T. pallidum* by the GUMP-ELAHA assay but positive for antibody elicited by treponemal infection, 8 had previous clinical diagnoses of syphilis. Accounting for the new information increased the sensitivity and specificity values of the *T. pallidum* component (table 3; values are in square brackets). No previous results or relevant clinical histories were available for the remaining 4 subjects. No dual infections were detected among the GUMP targets; however, a total of 8 subjects contained indications of 2 microbes in their specimens. Three subjects were positive for *Chlamydia trachomatis* and *T. pallidum;* 2 subjects were positive for *C. trachomatis* and *Neisseria gonorrhoeae;* 2 subjects were positive for *T. pallidum* and HSV-2; and 1 subject was positive for *T. pallidum* and *N. gonorrhoeae.*

In all but 3 (5.0%) of the subject specimens tested, the endogenous internal control template was amplifiable. Clinically, the failure of the endogenous internal control only affected the diagnostic outcome of 2 (3.3%) of the study specimens, because one of the endogenous retrovirus–negative samples was positive for a pathogen.

C. (Klebsiella) granulomatis *confirmatory assays.* The *C. granulomatis* PCR primers yielded a 328 base pair amplicon from *C. granulomatis* and other *Klebsiella* species, including *K. pneumoniae, K. oxytoca* species, and *K. planticola,* and from some *E. coli* strains. Discrimination of these from true *C. granulomatis* templates was achieved by generating a diagnostic 167–base pair fragment following restriction of the amplicon with *Hae*III (figure 2). No *C. granulomatis* was detected during the prospective study period; however, ongoing use of GUMP detected *C. granulomatis* in 1 instance during 2004 and in 2 instances during 2005 from clinically diagnosed subjects. DNA sequencing of a fragment of the 16S rRNA gene of the 3 strains confirmed that they shared 100% of their nucleotide sequence identities. Their nearest homologues were *C. granulomatis* (99.5% identity) and *Klebsiella rhinoscleromatis* (99.3% identity). Phylogenetic analysis confirmed these associations, dem-

Table 2. Results of genital ulcer disease multiplex PCR sensitivity studies using multiplex PCR reactions and synthetic templates either individually or in a coamplified pool.

	Copies per reaction		
Target	3 (3×10^0)	30 (3×10^1)	⩾300 (3×10^2)
pWC*hsv1*			
Single template	+	+	+
Pooled template	−	+	+
pWC*hsv2*			
Single template	+	+	+
Pooled template	+	+	+
pWC*tpal*			
Single template	+	+	+
Pooled template	−	+	+
pWC*hd*			
Single template	+	+	+
Pooled template	+	+	+
pWC*cg*			
Single template	+	+	+
Pooled template	−	+	+

NOTE. +, Positive amplification; −, negative amplification.

Table 3. Results of the genital ulcer disease multiplex PCR (GUMP)–enzyme linked amplicon hybridization assay (ELAHA) study and the resulting assay performance indicators.

Target	No. of GUMP-positive specimens (no. of subjects)	No. of GUMP-negative, traditional test–positive specimens (no. of subjects)	No. of GUMP-negative specimens (no. of subjects)	No. of GUMP codetections	Sensitivity, %	Specificity, %	Positive-predictive value, %	Negative-predictive value, %
HSV-1	1 (1)	0	63 (54)	0	100	100	100	100
HSV-2	3 (3)	4 (3)	57 (49)	1 × *Treponema pallidum* RPR 1 × *T. pallidum* TPPA+/RPR−	64	100	100	93
T. pallidum	18 (14)	11 (11)	35 (30)	3 × *Chlamydia trachomatis*	70 [86]	93 [94]	90 [86]	78 [94]
Haemophilus ducreyi	0 (0)	0 (0)	64 (55)	NA	NA	NA	NA	NA
Calymmatobacterium granulomatis	0 (0)	0 (0)	64 (55)	NA	NA	NA	NA	NA

NOTE. *T. pallidum* assay performance values, adjusted for previous diagnoses, are listed in square brackets. Calculations were performed using specimen numbers. Sensitivity = [traditional assay positives / (traditional assay positives + GUMP negatives)]. Specificity = [traditional assay negatives / (traditional assay negatives + GUMP positives)]. Positive-predictive value = [(traditional assay positives / (traditional assay positives + GUMP positives)]. Negative-predictive value = [(traditional assay negatives / (traditional assay negatives + GUMP negatives)]. HSV, herpes simplex virus; NA, not applicable; RPR, rapid plasmin reagin; TPPA, *T. pallidum* particle agglutination.

onstrating branching patterns similar to those described elsewhere (figure 3) [5, 9].

DISCUSSION

The GUMP assay was developed to detect sexually transmitted ulcerating agents among at-risk Australian populations, with a view to expediting the eradication of these diseases from Australia. The assay is currently in restricted use in Northern Queensland, which resulted in 3 instances in which *C. granulomatis* was detected, confirming that donovanosis remains in some parts of Australia. The lack of *H. ducreyi* detections suggest that chancroid is uncommon among our subjects; however, testing a larger specimen population will be required to translate this suggestion to the broader target community.

GUMP-ELAHA proved to be sensitive, even when all 5 microbial templates were coamplified; however, it is extremely unlikely that this would occur in a clinical environment. Our expanded assay retained identical analytical sensitivity to the multiplex PCR described by Orle et al. [23], on which GUMP-ELAHA was founded. Primers used in the assay used by Orle and colleagues were further validated by use in India, Thailand, and Lesotho [1, 20, 24]. The improvements to sensitivity and specificity brokered by the ELAHA satisfactorily balanced the addition of primers to detect *C. granulomatis* and an endogenous retrovirus–3 target serving as an endogenous internal control. Additionally, because GUMP is capable of codetecting a range of templates at a range of concentrations from a single reaction, it follows that clinical samples that are strongly positive for a single microbial template do not require further investigation to ensure that competitive PCR conditions have not masked the amplification of a second or third target.

Our study included, for the first time in a multiplex PCR assay to detect agents of GUD, an endogenous internal control to monitor template quality and purification efficiency. Amplification failure occurred for 5.0% of all specimens. This was similar to previous reports of diagnostic PCR failure [2, 11, 17, 25, 28]. Nonetheless, only 3% of the field trial results were adversely affected, which was less than that reported by Orle et al. [23], and we assumed that PCR inhibition or template degradation was the cause of these failures. Additional specimens were not available to permit re-extraction of endogenous internal control–negative material.

The analyses of sensitivity and specificity data for the *T. pallidum* component of the assay were affected by the nature of the conventional testing methods and a paucity of detailed clinical histories. Lesion swabs were the specimen type of choice for the GUMP assay to detect microbes at the site of infection; however, traditional testing for syphilis principally relied on serological assays, which detect a nonspecific immune response to recent, previous, and systemic treponemal infection.

The *T. pallidum* primers theoretically amplify other members of the *T. pallidum* species, because of their genetic similarities; however, the nonvenereal disease associated with these other treponemes is uncommon in Australia and generally responds to the therapies used to treat syphilis. The *C. granulomatis* component of the multiplex PCR was the source of the only proven unintended amplification observed during use of GUMP-ELAHA. Nonetheless, restriction-enzyme digestion and nucleotide sequencing adequately discriminated between *C. granulomatis* and genetically similar bacteria. Diagnostic amplification of *C. granulomatis* DNA by PCR was first described in an article in 1999 by Carter et al. [7], in which the *phoE* (phosphate porin) gene was targeted . This assay was complemented by nucleotide sequencing to identify 2 unique base

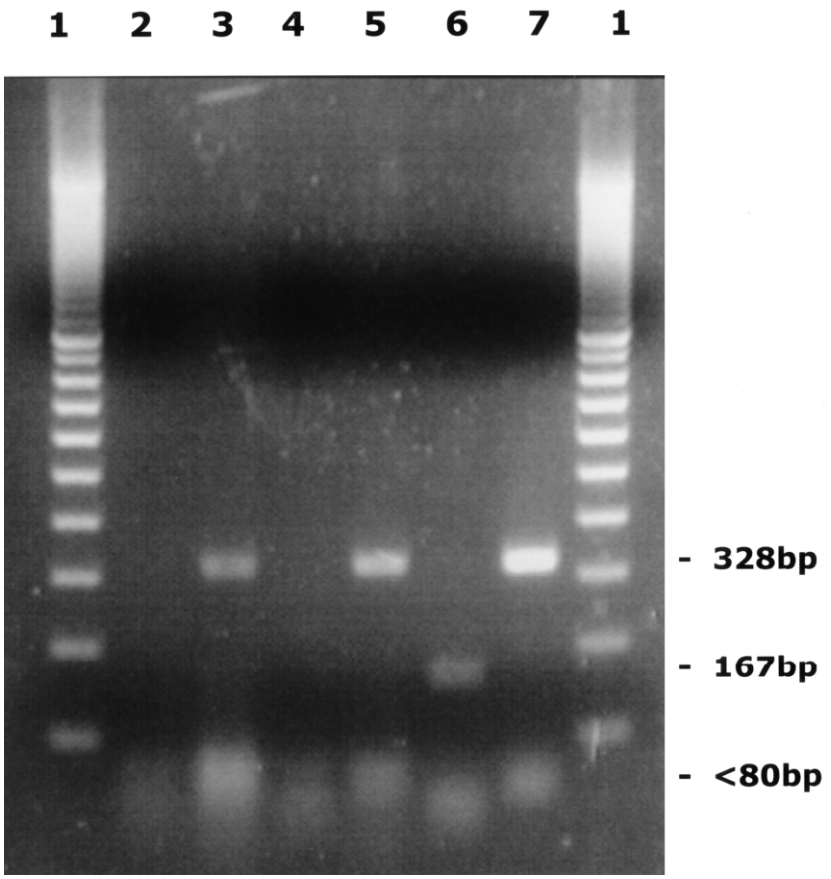

Figure 2. Agarose gel electrophoresis of *Klebsiella* species and suspected *Calymmatobacterium granulomatis* amplicon before and after *Hae*III restriction digestion. *Lane 1,* 100–base pair (bp) DNA molecular weight marker; *lanes 2* and *3, Escherichia coli* amplicon following and preceding restriction digestion, respectively; *lanes 4* and *5, Klebsiella pneumoniae* amplicon following and preceding restriction digestion, respectively; *lanes 6* and *7, Calymmatobacterium granulomatis* amplicon following and preceding restriction digestion, respectively.

changes in *C. granulomatis* that eliminated *HaeIII* restriction sites and differentiated this bacterium from other members of the *Enterobacteriaceae* family. Whereas Carter et al. [7, 10] used known positive biopsy samples to validate PCR sensitivity, together with a small number of genital swabs obtained from patients with other conditions to check specificity, we applied a synthetic control and examined DNA from numerous related microorganisms to extend the assay validation data, particularly for *H. ducreyi* and *C. granulomatis,* for which limited positive clinical material was available. Limited sequence data is publicly available for *C. granulomatis* strains; therefore, future studies should aim to sequence the *pho*E target to ensure the stability of the nucleotide polymorphism over time and around the world. Should the mutation prove to be a reliable target, a real-time PCR assay capable of discriminating the polymorphism should prove to be a useful and significantly more rapid diagnostic tool. Three suspected clinical cases of donovanosis were confirmed by GUMP-ELAHA and by restriction, indicating that the relevant polymorphism remains stable in Australia. Phylogenetic analysis of 16S rRNA gene sequencing indicated that the 3 Australian strains were most closely related to *C. granulomatis*. Restriction of the amplicon proved to be the most rapid confirmatory assay, whereas nucleotide sequencing was not essential for routine confirmation.

The GUMP study detected the presence of a target microbe in 34% of specimens provided to our laboratory. By comparison, traditional PCR, culture, and serological testing were able to identify at least 1 pathogen in 67% of specimens (64% of patients). Other studies report that more than one-third of specimens have negative results in multiplex PCR studies; however, the etiology of GUD in different study regions may vary dramatically, making comparison with our population difficult [6, 20, 24]. The disparity between GUMP-ELAHA and traditional results can be largely explained by the inclusion *C. trachomatis* and *N. gonorrhoeae* PCR and culture testing, in addition to the use of traditional serological assays that overdiagnosed *T. pallidum* infections; indeed, on retrospective analysis of the available diagnostic data, it was found that some subjects in this study had been previously diagnosed with syphilis. It is likely that these circulating antibodies contributed to

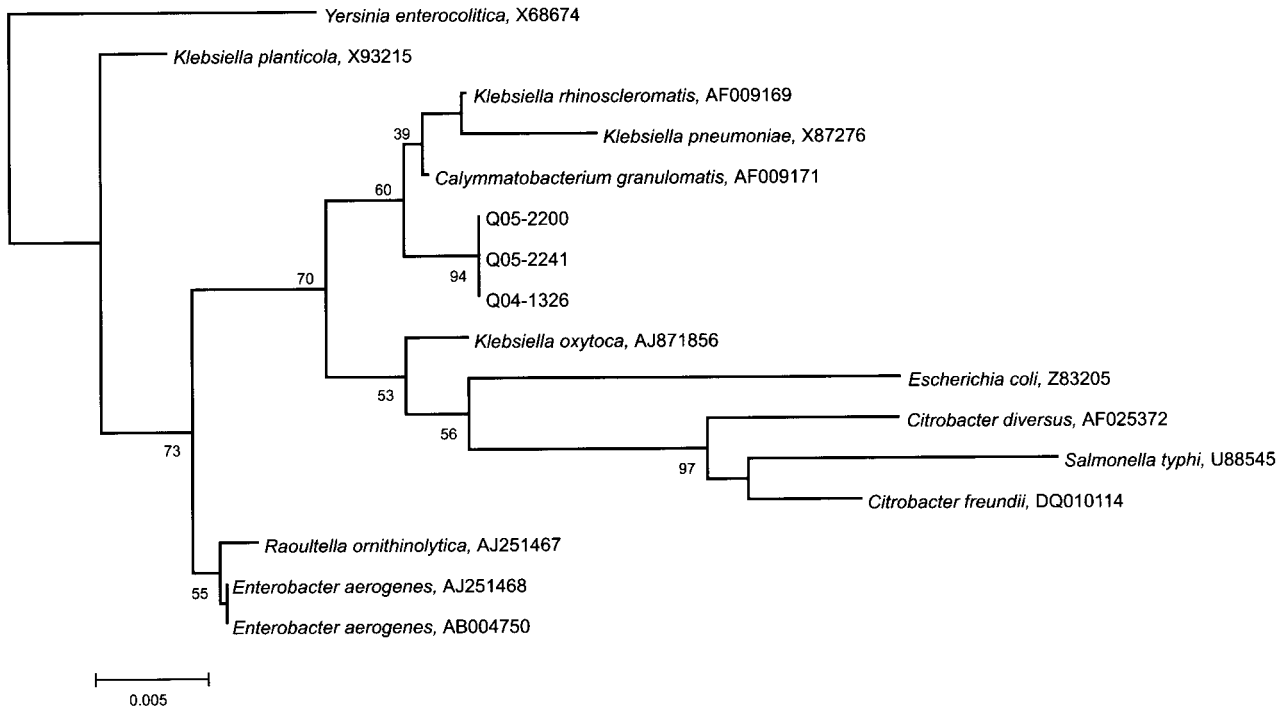

Figure 3. Phylogenetic analysis of suspected *Calymmatobacterium (Klebsiella) granulomatis* strains detected in Queensland (Q) during 2004 and 2005, presented on a topology tree prepared using Molecular Evolutionary Genetics Analysis 3 software [15] (GenBank accession numbers DQ112321-3). A 490–base pair 16S rRNA nucleotide alignment comprising Queensland strains together with other related and unrelated bacteria was prepared using BioEdit software, version 7.0. The nucleotide distance matrix was generated using the Kimura 2-parameter estimation. Nodal confidence values indicate the results of boot strap resampling ($n = 1000$). Accession numbers for sequences obtained from GenBank are shown.

a positive serodiagnosis, despite the absence of treponemes at the lesion site, as indicated by a negative GUMP result. Similar problems have been reported by Orle et al. [20], who proposed that treponemal serologic testing could lead to overdiagnosis of primary syphilis, reflecting its insensitive and nonspecific nature. The sensitivity and rapidity of GUMP-ELAHA could prove that it is especially useful for early identification of primary syphilis, whereas indirect RPR, fluorescent treponemal antibody absorption, TPPA, and Venereal Disease Research Laboratory assays can take weeks to be of diagnostic value. Similarly, Chen et al. [12] demonstrated no concordance between *H. ducreyi* serologic testing and PCR. Because GUMP-ELAHA was intended to detect microbial nucleic acids at the site of an active lesion, similar to the function of fluorescent microscopy, detailed comparison of the PCR with serological treponemal testing is largely unwarranted. However, the role of serological assays is important, because they provide an indication of the history of treponemal infection among persons with previous exposure; thus, serologic testing should continue to be used in conjunction with GUMP-ELAHA. We believe that the number of diagnoses could be further increased if complete clinical histories were available to address the relatively low number of HSV detections in our study. An unidentifiable number of the study subjects were suffering from recurring or persistent GUD and had been tested previously for HSV and subsequently prescribed treatment. Therefore, the overall likelihood that HSV could be detected among the GUMP study population was reduced, compared with other studies. A separate validation of the HSV component of GUMP-ELAHA demonstrated high sensitivity and specificity, reinforcing that presampling therapies could have been responsible for the reduced number of HSV detections. The GUMP population contained a total of 8 codetections; however, the severity of disease associated with suspected multiple infections could not be inferred from the data.

We have described a single-tube multiplex PCR assay to diagnose common and rare causes of GUD. GUMP performed with high sensitivity and was more rapid and more objective than conventional assays, including culture and fluorescent antibody techniques, for the direct detection of all targets. Additional applications of GUMP-ELAHA would benefit from a longer-term study of the role of PCR versus traditional testing among a larger and better-defined study population. Nonetheless, decreased turnaround times for results should permit earlier and more appropriate treatment of disease, whereas the less invasive nature of the sampling techniques should encourage more subjects to present for diagnosis of ulcerated lesions, thus improving our understanding of the epidemiology

of these diseases and helping us to focus our efforts to eradicate them.

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Potential conflicts of interest. All authors: no conflicts.

References

1. Beyrer C, Jitwatcharanan K, Natpratan C, et al. Molecular methods for the diagnosis of genital ulcer disease in a sexually transmitted disease clinic population in Northern Thailand: predominance of herpes simplex virus infection. J Infect Dis **1998**; 178:243–6.
2. Biel SS, Held TK, Landt O, et al. Rapid quantification and differentiation of human polyomavirus DNA in undiluted urine from patients after bone marrow transplantation. J Clin Microbiol **2000**; 38:3689–95.
3. Bowden FJ, Bright A, Rode JW, D Brewster. Donovanosis causing cervical lymphadenopathy in a five-month-old boy. Pediatr Infect Dis J **2000**; 19:167–9.
4. Bowden FJ, Savage J. Is the eradication of donovanosis possible in Australia? Aust N Z J Public Health **1998**; 22:7–9.
5. Boye K, DS Hansen. Sequencing of 16S rDNA of *Klebsiella:* taxonomic relations within the genus and to other *Enterobacteriaceae*. Int J Med Microbiol **2003**; 292:495–503.
6. Bruisten SM, Cairo I, Fennema HA, et al. Diagnosing genital ulcer disease in a clinic for sexually transmitted diseases in Amsterdam, the Netherlands. J Clin Microbiol **2001**; 39:601–5.
7. Carter J, Bowden FJ, Sriprakash KS, Bastian I, Kemp DJ. Diagnostic polymerase chain reaction for donovanosis. Clin Inf Dis **1999**; 28: 1168–9.
8. Carter JS, Hutton KS, Sriprakash DJ, et al. Culture of the causative organism of donovanosis *(Calymmatobacterium granulomatis)* in HEp-2 cells. J Clin Microbiol **1997**; 35:2915–7.
9. Carter JS, Bowden FJ, Bastian I, Myers GM, Sriprakash KS, Kemp DJ. Phylogenetic evidence for reclassification of *Calymmatobacterium granulomatis* as *Klebsiella granulomatis* comb. nov. Int J Syst Bacteriol **1999**; 49:1695–700.
10. Carter JS, Kemp DJ. A colorimetric detection system for *Calymmatobacterium granulomatis*. Sex Transm Infect **2000**; 76:134–6.
11. Cassinotti P, Siegl G. A nested-PCR assay for the simultaneous amplification of HSV-1, HSV-2, and HCMV genomes in patients with presumed herpetic CNS infections. J Virol Methods **1998**; 71:105–14.
12. Chen C-Y, Mertz KJ, Spinola SM, Morse SA. Comparison of enzyme immunoassays for antibodies to *Haemophilus ducreyi* in a community outbreak of chancroid in the United States. J Infect Dis **1997**; 175: 1390–5.
13. Hart G. Donovanosis. Clin Inf Dis **1997**; 25:24–30.
14. Kharsany AB, Hoosen AA, Kiepiela P, Naicker T, Sturm AW. Growth and cultural characteristics of *Calymmatobacterium granulomatis*—the aetiological agent of granuloma inguinale (donovanosis). J Med Microbiol **1997**; 46:579–85.
15. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **2001**; 17:1244–5.
16. Lewis DA. Chancroid: clinical manifestations, diagnosis, and management. Sex Transm Inf **2003**; 79:68–71.
17. Maass M, Dalhoff K. Comparison of sample preparation methods for detection of *Chlamydia pneumoniae* in bronchoalveolar lavage fluid by PCR. J Clin Microbiol **1994**; 32:2616–9.
18. Mackay IM, Gardam T, Arden KE, McHardy S, Whiley DM, Crisante E, Sloots TP. Co-detection and discrimination of six human herpesviruses by multiplex PCR-ELAHA. J Clin Virol **2003**; 28:291–302.
19. Mackay IM, Metharom P, Sloots TP, Wei MQ. Quantitative PCR-ELAHA for the determination of retroviral vector transduction efficiency. Mol Ther **2001**; 3:801–8.
20. Morse SA, Trees DL, Htun Y, et al. Comparison of clinical diagnosis and standard laboratory and molecular methods for the diagnosis of genital ulcer disease in Lesotho: association with human immunodeficiency virus infection. J Infect Dis **1997**; 175:583–9.
21. National notifiable diseases surveillance system. Annual reports: communicable diseases network Australia. **2005**. Available at: http://www.health.gov.au/internet/wcms/publishing.nsf/Content/cda-pubs-annlrpt-nndssar.htm. Accessed 12 April 2006.
22. O'Farrell N. Donovanosis. Sex Transm Inf **2002**; 78:452–7.
23. Orle KA, Gates CA, Martin DH, Body BA, Weiss JB. Simultaneous PCR detection of *Haemophilus ducreyi, Treponema pallidum,* and herpes simplex virus types 1 and 2 from genital ulcers. J Clin Microbiol **1996**; 34:49–54.
24. Risbud A, Chan-Tack K, Gadkari D, et al. The etiology of genital ulcer disease by multiplex polymerase chain reaction and relationship to HIV infection among patients attending sexually transmitted disease clinics in Pune, India. Sex Transm Dis **1999**; 26:55–62.
25. Rosenstraus M, Wang Z, Chang S-Y, DeBonville D, Spadoro JP. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J Clin Microbiol **1998**; 36:191–7.
26. Sartin JS. Molecular detection of *Treponema pallidum* via a 16S target. In: Persing DH, Smith TF, Tenover FC, White TJ, eds. Diagnostic molecular microbiology: principles and applications. Washington, DC: American Society for Microbiology, **1993**:230–4.
27. Syrmis MW, Whiley DM, Thomas M, et al. A sensitive, specific and cost-effective multiplex reverse-transcriptase–PCR assay for the detection of seven common respiratory viruses in respiratory samples. J Mol Diagn **2004**; 6:125–31.
28. Tong CYW, Donnelly C, Harvey G, Sillis M. Multiplex polymerase chain reaction for the simultaneous detection of *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Chlamydia psittaci* in respiratory samples. J Clin Pathol **1999**; 52:257–63.
29. Yuan CC, Miley W, Waters D. A quantification of human cells using an ERV-3 real time PCR assay. J Virol Methods **2001**; 91:109–17.

ERRATUM

In an article in the 15 May 2006 issue of the journal (Mackay IM, Harnett G, Jeoffreys N, Bastian I, Sriprakash KS, Siebert D, Sloots TP. Detection and discrimination of herpes simplex viruses, *Haemophilus ducreyi, Treponema pallidum,* and *Calymmatobacterium (Klebsiella) granulomatis* from genital ulcers. Clin Infect Dis 2006; 42:1431–8), several errors appeared in the author affiliations section. The corrected list of authors and their affiliations appears below.

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The journal regrets these errors.

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