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Evaluation of multiplex real-time PCR for detection of *Haemophilus ducreyi*, *Treponema pallidum*, herpes simplex virus type 1 and 2 in the diagnosis of genital ulcer disease in the Rakai District, Uganda

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Abstract

Objective: To develop a real-time PCR assay that reliably and accurately detects the predominant sexually transmitted aetiological agents of genital ulcer disease (GUD) (*Haemophilus ducreyi*, *Treponema pallidum* and herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2)) and to assess the use of real-time PCR diagnostic testing in a rural African field site.

Methods: Two multiplex real-time PCR reactions were used to detect *H ducreyi* and HSV-1/HSV-2 in ulcer swabs from 100 people with symptomatic genital ulcers in rural Rakai, Uganda. Results were compared with syphilis, HSV-1 and HSV-2 serology.

Results: Of 100 GUD samples analysed from 43 HIV positive and 57 HIV negative individuals, 71% were positive for one or more sexually transmitted infection (STI) pathogens by real-time PCR (61% for HSV-2, 5% for *T pallidum*, 3% for HSV-1, 1% for *H ducreyi* and 1% for dual *H ducreyi*/HSV-2). The frequency of HSV in genital ulcers was 56% (32/57) in HIV negative individuals and 77% (33/43) in HIV positive individuals ($p=0.037$). Assay reproducibility was evaluated by repeat PCR testing in the USA with 96% agreement ($\kappa=0.85$).

Conclusions: STI pathogens were detected in the majority of GUD swab samples from symptomatic patients in Rakai, Uganda, by real-time PCR. HSV-2 was the predominant cause of genital ulcers. Real-time PCR technology can provide sensitive, rapid and reproducible evaluation of GUD aetiology in a resource-limited setting.

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Contributors:

TRS, AH, AAT, BM, OL, CAG and SJR performed real-time PCR and serological testing and provided technical assistance on assay development. BM, PO and DS provided support for sample data and collection in Rakai, Uganda. TRS, AAT, OL, CAG, RHG, MJW, TCQ and SJR contributed to manuscript preparation and provided overall direction and support for the project.

Competing interests:

None.

Ethics approval:

The study was approved by the Science and Ethics Committee of the Uganda Virus Research Institute and the Western Institutional Review Board for Johns Hopkins University.

It is estimated that over 340 million new cases of sexually transmitted infections (STIs) occur annually throughout the world, with the highest number in developing countries.¹ These STIs frequently present as genital ulcer disease (GUD) of which the predominant aetiological agents are *Haemophilus ducreyi* (chancroid), *Treponema pallidum* (syphilis) and herpes simplex virus type 2 (HSV-2).

Genital herpes, mainly caused by HSV-2, is the leading cause of genital ulceration worldwide.²⁻⁵ Recent investigations indicate a rise in genital herpes caused by HSV-1.^{3,6,7} Primary HSV-1 and HSV-2 infections are clinically indistinguishable and require laboratory tests to differentiate.^{6,8}

GUD caused by HSV-2 is also a risk factor for HIV acquisition and transmission,^{2,9-11} and model estimates from sub-Saharan Africa suggest that the proportion of HIV incidence attributable to HSV-2 infection may increase as the HIV epidemic matures.^{9,12}

Standard methods used to detect the causative agents of GUD include in vitro culture, serology tests and microscopy,¹³⁻¹⁵ however, despite their potentially high sensitivity and specificity, these methods have limitations. In vitro culture of *H. ducreyi* is difficult and often impractical in clinical and research settings.¹³ Detection of HSV by culture varies greatly with duration and stage of the ulcerative lesion.¹³ Serological tests commonly used to diagnose syphilis and herpes do not detect infection during the early pre-seroconversion window or distinguish between active and latent infection.

Real-time PCR is a fast, sensitive and high-throughput method for pathogen detection and is rapidly becoming a mainstay of research and clinical diagnostic applications.¹⁶⁻¹⁸ Several PCR-based techniques that require post-amplification processing have been used to detect GUD pathogens.^{13,19,20} The fluorogenic probes used in real-time PCR enable direct measurement of product amplification as the reaction proceeds, minimising additional “hands-on” time and decreasing the risk of contamination. The probes permit sensitive and specific detection of target nucleic acids over a wide dynamic range of input sample concentrations.^{21,22} Furthermore, the use of multiple probes per reaction, or multiplexing, allows several pathogens to be detected in a single well.

We established two duplex real-time PCR reactions to detect *H. ducreyi*, *T. pallidum*, HSV-1 and HSV-2 in an on-site laboratory in Rakai, Uganda. We report the validation of this duplex real-time PCR assay and evaluate its use in this setting.

METHODS

Study population and clinical specimens

The population consisted of people enrolled in the Rakai Community Cohort who were seen during annual cohort surveys or in mobile clinics and who reported genital ulceration at time of interview. During the study period 2002–2006, approximately 120 consecutive clinical episodes of GUD were recorded among Rakai participants. Although a single swab was to be collected per episode, 100 swabs were delivered and stored in the laboratory during this period and used in this evaluation. Samples were collected in 1 mL specimen transport medium (Roche Amplicor STM, Roche Diagnostics, Indianapolis, USA) or Herptran medium (Perkin Elmer, Waltham, Maryland, USA) and stored at –80°C. For most patients (98%), blood samples were also collected at the same visit and sera was stored at –80°C.

Real-time multiplex PCR

The real-time PCR methods presented here are based on previously established PCR assays that have been used to detect GUD pathogens^{13,23} but with two major adaptations. First, a

widely used two-step PCR assay (triplex PCR followed by colorimetric detection), which simultaneously detects *H ducreyi*, *T pallidum* and HSV,¹³ was optimised for use in the real-time PCR format. The original 5' primer biotinylation was removed. A unique fluorophore (VIC for HSV, TET for syphilis and FAM for chancroid) and 3' MGB quencher that enhances probe-target annealing were added to the probe. Tenfold dilutions of positive control DNA and varying primer (250–900 nM) and probe (100–250 nM) concentrations were used to determine optimal detection conditions. Using these modified primers, probes and Qiagen HotStar Taq DNA Polymerase kit (Qiagen, Valencia, California, USA), one or more pathogens (*H ducreyi*, *T pallidum* and HSV) were simultaneously detected in approximately 41% of swab specimens by real-time PCR (data not shown).

Second, to improve detection and distinguish between HSV-1 and HSV-2, HSV primers and probe were removed from the original reaction and a second separate reaction for HSV-1 and HSV-2 was added. The *H ducreyi/T pallidum* duplex reaction was re-evaluated for optimal primer (250 nM) and probe (100 nM) concentrations and used with ABI Master Mix (Applied Biosystems, Valencia, California, USA). The HSV-1/HSV-2 typing reaction is the same as previously reported for real-time PCR detection of HSV-1 and HSV-2 using the ABI 7900HT thermocycler²³ (Applied Biosystems) and ABI Master Mix. Thus, the real-time PCR assay presented here consists of two duplex PCR reactions: one that detects *H ducreyi* and *T pallidum* and the other that detects HSV-1 and HSV-2. Except for specific primers and probes, all PCR components and thermocycling parameters are identical for both reactions enhancing the efficiency of reaction assembly.

DNA was extracted from swab samples using the QIAamp DNA Blood Mini kit following the blood and body fluids protocol as supplied by the manufacturer (Qiagen). Four positive controls were diluted 1:2 in TE buffer and extracted: *H ducreyi* (ATCC # 51566 or 700724, Manassas, Virginia, USA), *T pallidum* (Lee Laboratories/Becton Dickinson # 210483, Grayson, Georgia, USA), HSV-1 strain GHSV-UL46 (ATCC # VR-1544) and HSV-2 strain MS (ATCC # VR-540). Two separate multiplex assays were performed for each sample: one for *H ducreyi/T pallidum* detection and the other for HSV-1/ HSV-2 detection. The primer and probe sequences used for this study have been previously reported.^{13 23}

The *H ducreyi/T pallidum* reaction contained ABI 2× Universal Master Mix, 250 nM of each of four primers (K07A, K08A, K03A, K014) and 100 nM of each of two probes (K015, K017). The HSV-1/HSV-2 reaction contained ABI 2× Universal Master Mix, 800 nM of each of two primers (GbTypF, GbTypR) and 100 nM of each of two probes (HSVgBTyp1, HSVgBTyp2). Assays were performed in 96-well plate format using an ABI 7900 HT Fast real-time PCR instrument with a 50 µL total reaction volume that included 10 µL of extracted sample or positive control DNA. Thermocycle parameters were the same for both duplex assays: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec, 60°C for 1 min. Samples were run in duplicate for each duplex assay. To analyse results, the threshold was selected based on positive and negative controls and used to determine a threshold-cycle value (C_T , the PCR cycle at which amplification signal crosses the threshold). A cutoff C_T value was not designated. Samples were counted as weakly positive if they had a C_T value greater than 36 and a fluorescence signal increase in the multicomponent view of at least 1000 units. Discrepant or weakly positive samples were re-evaluated in singleplex format using a single primer pair and probe corresponding to one pathogen.

Swab samples were re-extracted and analysed in the USA for independent confirmation of real-time PCR results. Sample processing procedures were identical to those reported above except that DNA extraction was performed using the Roche MagNA Pure robot (Roche Diagnostics, Indianapolis, USA) with the DNA I Blood Cell High Performance kit and protocol. Samples were analysed with the same criteria used in the Rakai laboratory.

Serology

Blood samples from 98 of 100 people with current GUD symptoms were collected at the same visit. Sera were screened for the presence of antibodies to *T pallidum* using the nontreponemal Tolidine Red Untreated Serum Test (TRUST, New Horizons Diagnostic Corporation, Columbia, Massachusetts, USA) assay. TRUST-positive samples were further evaluated by the *Treponema pallidum* Particle Agglutination Assay (TPPA) (Serodia-TP PA kit, Fujirebio Inc, Tokyo, Japan). Samples reactive on both the TRUST and TPPA assays were classified as *T pallidum* antibody positive.

Sera from 94 GUD patients were tested for HSV-2 antibodies using the Kalon ELISA (Kalon Biological Ltd, Guilford, UK) according to the manufacturer's protocol with minor modifications. Samples were run in duplicate and the mean index value was used to classify HSV-2 status. People with an index value <0.9 were classified as HSV-2 negative as recommended by the manufacturer. Based on prior evaluation of test performance in Ugandan sera, HSV-2 positive individuals were defined as an index value of ≥ 1.5 .²⁴ Samples with equivocal results (0.9–1.5) were repeated by Euroimmune Western blot (Euroimmune, Lubeck, Germany) and HSV-2 Western blot at the University of Washington (Seattle, Washington, USA).²⁵ Confirmatory University of Washington HSV-2 Western blots were performed for samples that were HSV-2 seronegative by Kalon and HSV-2 positive by real-time PCR. HSV-1 serostatus was determined using the Euroimmune Western blot test according to manufacturer recommendations.

For all patients, HIV infection status at the time of the GUD visit was established using two HIV enzyme immunosorbent assays (Vironostika HIV-1, Organon Teknika, Charlotte, North Carolina, USA; Cambridge Biotech, Worcester, Massachusetts, USA) and Western blot (HIV-1 Western Blot, BioMerieux-Vitek, St Louis, Missouri, USA) for discordant results.

Statistical analysis

Data were analysed using Microsoft Excel 2003 and Intercooled STATA version 10.0 (StataCorp, College Station, Texas, USA). To compare proportions of detectable swabs in the real-time PCR assay versus triplex and other PCR assays and HSV-2 seropositive serum samples, two-tailed z-tests were performed and the resulting p values are reported. To detect associations between HSV by real-time PCR and HIV infection, both Fisher exact and χ^2 tests were performed.

RESULTS

Using duplex real-time PCR reactions, one or more pathogens were detected in 71% of swab specimens collected from 100 people with symptomatic genital ulcers (fig 1 and table 1). Altogether, 61% of samples were positive for HSV-2, 5% for *T pallidum*, 3% for HSV-1, 1% each for *H ducreyi* and dual *H ducreyi*/HSV-2, and the remaining 29% were undetectable for all four STI pathogens.

Of 100 patients, 43 (20 females and 23 males) were HIV seropositive (table 1). Altogether, 77% (33/43) of HIV seropositive individuals had detectable HSV-1 or HSV-2 in their ulcer swabs compared with 56% (32/57) of HIV negative individuals ($p=0.037$).

To confirm results obtained in the Rakai Uganda laboratory, swab specimens were shipped to the USA and re-tested using the same methodology. The overall agreement of real-time PCR results was high: 100% for *T pallidum*, 98% for *H ducreyi*, 97% for HSV-1 and 89% for HSV-2 ($\kappa=0.85$) (results not shown). Except for two samples, all discrepancies arose in HSV testing. Two samples were negative for *H ducreyi* when tested in the Rakai laboratory but positive in the USA. All three discordant HSV-1 samples and ten out of eleven HSV-2 discordant samples

were weakly positive by multiplex real-time PCR in Rakai. These samples were confirmed as PCR positive in Rakai by inspection of the fluorescent signal increase in the multicomponent plot as well as when repeated in singleplex format. However, all 14 samples tested negative in the USA.

Of 94 specimens tested for serologic syphilis, 11 (12%) were TRUST positive and confirmed by TPPA (table 2). Five of these eleven samples (45%) were also *T pallidum* positive by real-time PCR. Of the six samples (55%) that were *T pallidum* PCR negative, five contained either HSV-1 or HSV-2 DNA by real-time PCR.

HSV-1 serology was performed using Western blot, with 88% (80/91) of serum samples showing reactivity. HSV-1 specific DNA was detectable in three of the ulcer swabs from seropositive patients.

Altogether, 73 of 95 serum samples tested (77%) were HSV-2 seropositive by the Kalon ELISA assay. HSV-2 DNA was detected in 50 (68%) of the corresponding ulcer swabs by real-time PCR. Of the remaining 23 HSV-2 seropositive PCR negative samples, HSV-1 or *T pallidum* DNA was detected in four ulcer swabs. Conversely, 9 out of 22 HSV-2 seronegative samples (41%) contained HSV-2-specific DNA as determined by multiplex PCR, possibly representing recent infections during the window period prior to development of HSV-2 antibodies. HSV-2 serology for eight of these nine samples was repeated by Western blot, which can identify seroconversions earlier than Kalon,²⁶ and HSV-2 antibodies were detected in two samples.

Key messages

- ▶ Multiplex real-time PCR can rapidly and reproducibly detect the four predominant genital ulcer disease (GUD) pathogens: *Haemophilus ducreyi*, *Treponema pallidum*, herpes simplex virus type 1 and 2.
- ▶ Using real-time PCR in a Ugandan laboratory, GUD pathogens were detected in the majority (71%) of samples collected from patients with symptomatic genital ulcers.
- ▶ The ability to detect recent infection and to diagnose the cause of current ulceration regardless of prior infection are distinct advantages of real-time PCR methodology.

Of the 29 patients with undetectable STI pathogens by real-time PCR, 18 (62%) were HSV-2 seropositive, 1 (3%) was *T pallidum*/HSV-2 dual seropositive, 8 (28%) had no detectable antibodies to HSV-2 or *T pallidum* and 2 (7%) were not tested (data not shown). There was no statistically significant association between HSV-2 seropositivity and detection of pathogen by real-time PCR ($p=0.346$).

DISCUSSION

We demonstrated the use of real-time PCR technology for the diagnosis of GUD aetiology in a resource-limited research setting. Among patients with current symptomatic genital ulcers, a STI pathogen was detected in 71% of swab samples by real-time PCR. This represents an improvement in detection over both the original triplex real-time PCR assay on the same specimens (41%; $p \leq 0.001$) (data not shown) and previous reports of PCR-based detection of GUD pathogens in Uganda (50–55%; $p \leq 0.003$)^{5,27} and other geographical regions.^{20,28,29}

Results obtained by duplex real-time PCR were highly reproducible upon repeat testing in US laboratories by independent technicians with an observed agreement of 96%. All discordant HSV results were positive in Rakai and negative upon repeat in the USA. While it is possible that the initial results were false positives, sample degradation (upon multiple freeze-thaw

cycles before and after shipping), different DNA extraction procedures and variation in laser calibration between the two instruments may have also contributed to these discrepancies. The majority of discrepant samples were initially weakly positive in Uganda, even upon repeat testing in singleplex reactions, suggesting that initial pathogen titres in these specimens were low.

In most resource-limited settings, GUD is syndromically managed. While this real-time PCR method does not currently play a role in clinical management, it can be used to rapidly discriminate between latent and active infection among seropositive individuals and therefore more accurately detect the aetiological agent causing ulceration. Real-time PCR can also identify early GUD infections in the pre-seroconversion window. As such, it can be a powerful tool for clinicians and researchers in sub-Saharan Africa to rapidly detect common GUD pathogens in their patient populations and map the changing epidemiology of the disease over time.

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Figure 1.
Aetiology of genital ulcers by multiplex real-time PCR.

Table 1
Patient demographics and distribution of *Haemophilus ducreyi* (HD), *Treponema pallidum* (TP), herpes simplex virus type 1 (HSV-1) and HSV-2 by multiplex real-time PCR

Genital ulcer disease aetiology by real-time PCR						
	HD n (%)	TP n (%)	HSV-1 n (%)	HSV-2 n (%)	HD/ HSV-2 n (%)	ND n (%)
Total (n=100)	1 (1)	5 (5)	3 (3)	61 (61)	1 (1)	29 (29)
Gender						
Male (n=50)	1 (2)	2 (4)	3 (6)	28 (56)	1 (2)	15 (30)
Female (n=50)	0 (0)	3 (6)	0 (0)	33 (66)	0 (0)	14 (28)
Age (y)						
19–29 (n=51)	0 (0)	3 (6)	0 (0)	33 (65)	1 (2)	14 (27)
30–39 (n=31)	0 (0)	1 (3)	0 (0)	20 (65)	0 (0)	10 (32)
40+ (n=15)	0 (0)	0 (0)	3 (20)	7 (47)	0 (0)	5 (33)
Not known (n=3)	1 (33)	1 (33)	0 (0)	1 (33)	0 (0)	0 (0)
HIV serostatus						
Negative (n=57)	1 (2)	4 (7)	2 (4)	29 (51)	1 (2)	20 (35)
Positive (n=43)	0 (0)	1 (2)	1 (2)	32 (74)	0 (0)	9 (21)

ND, pathogen not detected by real-time PCR.

Table 2
Comparison of multiplex real-time PCR with syphilis, HSV-1 and HSV-2 serology

	Syphilis serology		HSV-1 serology		HSV-2 serology				
	TRUST/TPPA		Euroimmune		Kalon				
	+	–	+	–	+	–			
	(%)	(%)	Total (%)	(%)	Total (%)	(%)	Total (%)		
PCR+	5 (45)	0 (0)	5 (5)	3 (4)	0 (0)	3 (3)	50 (68)	9 (41)	59 (62)
PCR–	6 (55)	83 (100)	89 (95)	77 (96)	11 (100)	88 (97)	23 (32)	13 (59)	36 (38)
Total	11 (100)	83 (100)	94 (100)	80 (100)	11 (100)	91 (100)	73 (100)	22 (100)	95 (100)

HSV, herpes simplex virus; TPPA, *Treponema pallidum* particle agglutination assay.