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A multicenter prospective trial to asses a new real-time polymerase chain reaction for detection of *Treponema pallidum*, herpes simplex-1/2 and *Haemophilus ducreyi* in genital, anal and oropharyngeal ulcers

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Abstract

Treponema pallidum, herpes simplex virus types 1 or 2 (HSV-1/2) and *Haemophilus ducreyi* are sexually transmitted pathogens that can cause genital, anal and oropharyngeal ulcers. Laboratory evaluation of these pathogens in ulcers requires different types of specimens and tests, increasing the risk of improper specimen handling and time lapse until analysis. We sought to develop a new real-time PCR (TP-HD-HSV1/2 PCR) to facilitate the detection of *T. pallidum*, HSV-1/2 and *H. ducreyi* in ulcers. The TP-HD-HSV1/2 PCR was tested (i) in a retrospective study on 193 specimens of various clinical origin and (ii) in a prospective study on 36 patients with genital, anal or oropharyngeal ulcers (ClinicalTrials.gov # NCT01688258). The results of the TP-HD-HSV1/2 PCR were compared with standard diagnostic methods (*T. pallidum*: serology, dark field microscopy; HSV-1/2: PCR; *H. ducreyi*: cultivation). Sensitivity and specificity of the TP-HD-HSV1/2 PCR for *T. pallidum* were both 100%, for HSV-1 100% and 98%, and for HSV-2 100% and 98%, respectively. *T. pallidum* and HSV-1/2 were detected in 53% and 22% of patients in the prospective study; *H. ducreyi* was not detected. In the prospective study, 5/19 (26%) specimens were true positive for *T. pallidum* in the TP-HD-HSV1/2 PCR but non-reactive in the VDRL. The TP-HD-HSV1/2 PCR is sensitive and specific for the detection of *T. pallidum* and HSV-1/2 in routine clinical practice and it appears superior to serology in early *T. pallidum* infections.

Keywords: Genital ulcer disease, *Haemophilus ducreyi*, herpes simplex virus, real-time PCR, syphilis, *Treponema pallidum*
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Introduction

Treponema pallidum, herpes simplex virus types 1 or 2 (HSV-1/2) and *Haemophilus ducreyi* are, worldwide, the most common sexually transmitted pathogens that cause ulcers of the genital, anal and oropharyngeal region [1,2]. The global annual incidence of ulcers caused by sexually transmitted infections is 20 million cases [3], and genital ulcers are a significant risk factor for transmission and acquisition of human immunodeficiency virus (HIV). Identification of ulcer-causing pathogens is a prerequisite for an effective therapy and reducing the risk of HIV infection [4], but is unreliable based on clinical presentation due to pleomorphic clinical presentation and mixed infections [1,5,6]. Hence, laboratory methods are indispensable for substantiating the diagnosis. Currently, the most widely used standard methods ('reference methods') for the diagnosis of *T. pallidum* infection are serology and dark field microscopy [7,8]; for HSV-1/2 infection these are PCR, cultivation, serology and direct immunofluorescence (DIF) [8–10]; for *H. ducreyi* infection it is cultivation [8].

However, these methods have limitations. For example, *T. pallidum* serology has a low sensitivity in early syphilis when ulcers occur [11]. Dark field microscopy is prone to improper specimen collection and handling, dark field microscopes are not widely available, evaluation requires an experienced observer, and this method is not applicable for oral specimens [12,13]. For the detection of HSV-1/2, PCR has increased the diagnostic sensitivity by almost 70% compared with cultivation [8,9]. The laborious and poorly sensitive HSV cultivation needs 7–10 days to exclude an infection, and sensitivity and specificity of serology are insufficient during early infection [14]. *H. ducreyi* is a fastidious pathogen and cultivation has a sensitivity of <80% and is not suitable for routine clinical practice [15]. As a consequence, laboratory evaluation of genital ulcers requires the collection of various specimen types and the coordinated distribution to different laboratories, increasing the risk of improper specimen handling and time lapse until analysis. This may have implications for sensitivity, and may delay the diagnosis and specific treatment. To overcome these difficulties, we developed a new real-time PCR (TP-HD-HSV1/2 PCR) for the diagnosis of *T. pallidum*, HSV 1/2 and *H. ducreyi* (TP-HD-HSV1/2 PCR) in genital, anal and oropharyngeal ulcers. Nylon flocked swabs were used to collect specimens from the ulcer base, which is an appropriate method for detecting *T. pallidum*, HSV-1/2 and *H. ducreyi* by PCR [16,17].

We sought to investigate (i) the sensitivity, specificity and clinical applicability of the TP-HD-HSV1/2 PCR and (ii) the rate of pathogens and clinical characteristics in patients with ulcers in a prospective clinical study. This study was designed and performed according guidelines for the evaluation and reporting of new diagnostic tests [18,19].

Materials and Methods

TP-HD-HSV1/2 PCR

New primers and probes targeting the 16S rRNA gene were designed for *T. pallidum* and *H. ducreyi* (Table 1). Oligos were designed with Primer3 (v. 0.4.0) [20] based on GenBank sequences M88726.1 (*T. pallidum*) and M75078.1 (*H. ducreyi*) and commercially synthesized (Microsynth AG, Balgach, Switzerland). HSV-1/2 specific oligos have been described previously [21]. Optimal reaction conditions were assessed by amplification of tenfold dilutions of DNA with increasing annealing temperature from 50 to 66°C. DNA from *T. pallidum* was extracted from rabbit testis homogenate with the DNeasy tissue kit (Qiagen, Hilden, Germany) and provided by Euroimmun AG (Lübeck, Germany), HSV-1/2 DNA was provided by Professor Werner Kempf (Zurich, Switzerland), and *H. ducreyi* DNA was isolated from strain CIP 100296 (CRBIP, Institute Louis Pasteur, Paris, France). Prior tenfold dilutions, the concentrations of genomic DNA stock were determined with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wohlen, Switzerland) and were as follows (average of three measurements): *T. pallidum*, 30.7 ng/μL; HSV-1, 7.9 ng/μL; HSV-2, 10.4 ng/μL; *H. ducreyi* 44.9 ng/μL. Species identity of amplicons was confirmed by sequencing (Microsynth AG).

TABLE 1. Primers and probes used in the new real-time multiplex PCR

Name[a]	Sequence	Tm[b]
Treponema pallidum[c]		
TP-Zh-131-f	GCCTTTGAGATGGGGATAGC	59.2
TP-Zh-245-r	GTCGCAGGCTCATCTCTGA	58.9
TP-Zh-220-p	FAM-CCGCAGCCCCTTTCCT CTCA-BHQ-1	67.9
Internal control		
IC-Zh-p	Yakima Yellow-TCGTGCCTCA GTGCCAGTCAC-BHQ-1	66.3
Haemophilus ducreyi[d]		
HD-Zh-992-f	ACATCCATAGAAGAACTCAGAGATGA	60.3
HD-Zh-1150-r	TTGAGTTCCCATCAYTACATGCT	59.9
HD-Zh-1022-p	Yakima Yellow-GTGCCTTCGGGAA CTATGTGACAGGT-BHQ-1	67.6
Herpes simplex 1/2[e]		
GbTypF	CGCATCAAGACCACCTCCTC	60.3
GbTypR	GCTCGCACCACGCGA	55.2
GbTyp1	FAM-TGGCAACGCGGCCCAAC-BHQ-1	60.2
GbTyp2	ROX-CGGCGATGCGCCCCAG-BHQ-2	60.8

Tm, melting temperature.
[a]Numbers refer to the oligo 5′ start position in the target sequence; f indicates forward primer; p, probe; r, reverse primer.
[b]Average of 4 Tm calculators (Primer3, Microsynth, OligoCalc and Oligo Analyzer) in degrees Celsius.
[c]Target gene was the bacterial 16S ribosomal RNA gene, GenBank M88726.1.
[d]Target gene was the bacterial 16S ribosomal RNA gene, GenBank M75078.1.
[e]Corey *et al.* [21].

For DNA extraction, 0.5 mL of swab eluat was spinned at 20 000 × *g* for 10 min. The pellet was incubated with 40 μg proteinase K (Roche Diagnostics, Rotkreuz, Switzerland) for 2 h at 54°C on a shaking incubator. Proteinase K was inactivated for 10 min at 94°C, detritus was pelleted at 20 000 × *g* for 10 min at room temperature and the DNA-containing supernatant was stored at −20°C until amplification.

PCR was performed with a Roche LightCycler 2.0 (Roche Diagnostics). The assay was split into two reactions (i.e. tube 1 for detecting *T. pallidum* and the internal control (inhibition control) and tube 2 for *H. ducreyi*, HSV-1 and HSV-2). The 20-μL reaction volume contained 4 μL 5 × LightCycler Taq-Man mastermix (Roche), 0.25 μL LightCycler Uracil-DNA Glycosylase, 0.5 μM of each primer, 0.1 μM probe, 1 μL internal control (only in tube 1) and 5 μL extracted DNA. Reaction conditions were: activation of uracil-N-glucosylase at 40°C for 10 min, pre-denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 10 s and annealing/amplification at 66°C for 1 min. A colour compensation according to the LightCycler 2.0 manufacturer's instructions was performed and used for valid interpretation of results.

Testing for analytical sensitivity and specificity

Analytical sensitivity was determined with serial dilutions of pathogen-specific DNA fragments synthesized in pUC57 plasmids (GenScript, Piscataway, NJ, USA). The internal control (inhibition control) was based on the *T. pallidum* target sequence with a unique probe-binding region. Serial dilutions of plasmid DNA were analysed in three independent runs. Although being a commonly used method, the DNA copy numbers determined by PCR may differ between plasmid vs. chromosomally encoded sequences such as the pathogen-specific sequences targeted in this study [22]. Also, inhibitory compounds present in clinical specimens such as lactoferrin or haemoglobin [23] may alter the PCR kinetics compared with the use of recombinant plasmid DNA.

Analytical specificity was confirmed (i) against published DNA sequences of human, bacterial or viral origin with the NCBI BLASTN algorithm (query database: 'nucleotide collection') and with Primer BLAST (query database: 'nr' with no restriction to organisms) [24], (ii) by sequencing the amplicons for each pathogen, and (iii) by assessing possible cross-reactions of primers and probes with human DNA and inhibitory effects of human DNA on the PCR reaction.

Testing for clinical sensitivity and specificity

We tested two panels of specimens from genital, anal or oropharyngeal ulcers with the new TP-HD-HSV1/2 PCR.

1. *The retrospective study* comprised: (i) 115 DNA extracts from clinical specimens (25 cervical swabs, 25 urethral swabs, 11 vulva swabs, four penis swabs, two ulcer swabs, nine blister swabs, one anal swab, one vaginal swab, 11 respiratory tract swabs, 10 faeces samples and 16 urine samples), which were collected at Bioanalytica AG, Lucerne, Switzerland; these samples originated mainly from patients with urogenital disorders and a few with respiratory or gastrointestinal disorders and were chosen in order to assess the specificity; (ii) 18 DNA extracts from genital ulcer swabs; and (iii) 60 swab eluates from genital ulcer swabs (cotton swabs in phosphate-buffered saline collected at the Public Health Laboratory in Amsterdam, the Netherlands).
2. *The prospective clinical study* comprised swabs from patients with genital, anal or oropharyngeal ulcers. The study was approved by the ethics committees in Zurich, Switzerland, and Graz, Austria (ClinicalTrials.gov # NCT01688258). Patients were recruited at outpatient clinics in Switzerland (Department of Dermatology, University Hospital Zurich; Division of Infectious Diseases, University Hospital Zurich; Division of Dermatology, City Hospital Triemli, Zurich;) and Austria (Department of Dermatology and Venereology, Medical University of Graz). All patients gave written informed consent and were seen by a board-certified dermatologist or infectious diseases specialist at enrollment. Inclusion criteria were (i) presence of genital, anal or oropharyngeal ulcers, (ii) patient's age $\geq$18 years, and (iii) written informed consent. Exclusion criteria were systemic or topical antibiotic therapy within 2 weeks before enrollment. Clinical specimens for the TP-HD-HSV1/2 PCR were taken from the ulcer base with nylon flocked swabs (eSwabs; Copan Diagnostics, Brescia, Italy) and stored at -20°C until DNA extraction and PCR at the Department of Dermatology, University Hospital Zurich. A case report form with clinical metadata was available for each patient.

Comparative diagnostics with reference methods

In the retrospective panel, a reference PCR was performed in 78/193 specimens (40%) for *T. pallidum* [25] and in 104/193 (54%) for HSV-1/2 [26,27]. *H. ducreyi* was not tested. Specimens not tested by reference PCR were assumed to be negative. In the prospective study, the results of the TP-HD-HSV1/2 PCR were compared with the following reference methods: for *T. pallidum* dark field microscopy and syphilis serology as previously described [11]; for HSV-1/2 a validated routine PCR (Institute of Medical Virology, University of Zurich; Molecular Biology, Department of Dermatology, Medical University of Graz); and for *H. ducreyi* cultivation on Chocolate II Agar (BD, Allschwil, Switzerland) and Mueller-Hinton-Agar containing horse blood and IsoVitaleX and GC Hemoglobin Agar containing calf serum and IsoVitaleX [28].

In cases with discrepant results, samples were sent to a reference laboratory in Amsterdam, the Netherlands (Public Health Laboratory, GGD Amsterdam) for re-analysis. There, a PCR specific for *T. pallidum* described by Koek *et al.* [25] or HSV-1/2 described by van Doornum *et al.* [26] were used. Both PCR assays were routinely used for the detection of the respective pathogen in this reference laboratory.

Statistics

Statistical analysis was performed with R statistical software, version 3.0.1 (Vienna, Austria) [29]. Sensitivity and specificity of the new TP-HD-HSV1/2 PCR were computed with the function 'epi.tests'. The McNemar test (function 'mcnemar.test') was applied to assess differences between the TP-HD-HSV1/2 PCR and reference methods. Continuous variables were tested for parametric distribution by histograms, quantile-quantile plots and the Shapiro test. Differences between groups were tested with the Kruskal–Wallis rank sum test, ANOVA or Fisher's exact test where applicable. In the case of a p value <0.05, a pairwise comparison between all groups was computed with the Wilcoxon rank sum test,

ANOVA or Fisher's exact test where applicable. Adjustment for multiple comparisons ($n = 3$) was computed with the method of Benjamini and Hochberg (function 'p.adjust', method = 'fdr') or TukeyHSD (function 'TukeyHSD') where applicable.

Results

Analytical sensitivity and specificity of the TP-HD-HSV1/2 PCR

The sensitivity to detect a pathogen was NOT lower if DNA of another pathogen was present in the reaction mix. Also, human DNA was not detected by the TP-HD-HSV 1/2 PCR and the presence of human DNA had no influence on the sensitivity of the new PCR. The analytical sensitivity was ≤10 plasmid copies/reaction for *T. pallidum* and *H. ducreyi*, and ≤25 plasmid copies/reaction for the HSV assay.

Retrospective study

Results for *T. pallidum* were 100% concordant between the TP-HD-HSV1/2 PCR and the reference *T. pallidum*-specific PCR (Tables 2 and 3). In the TP-HD-HSV1/2 PCR, 2/193 reference samples (1%) were considered false-positive for HSV-1 and 3/193 (2%) for HSV-2 (Table 2). These samples were positive with high Ct values (range, 34.4–39.5 cycles), indicating a very low amount of HSV-specific DNA supposedly due to cross-contamination during DNA extraction. *H. ducreyi* was not tested in the reference laboratories and no sample was positive in the TP-HD-HSV1/2 PCR.

Prospective clinical study

Patients. From 36 patients with genital, anal or oropharyngeal ulcers, 39 specimens were collected between July 2012 and June 2013. According to reference methods, 17/36 patients (47%) were infected with *T. pallidum,* 3/36 (8%) with HSV-1 and 4/36 (11%) with HSV-2. Upon discrepancy analyses, 19/36 patients (53%) had *T. pallidum*, and 4/36 patients (11%) each had HSV-1 and HSV-2 (Table 4). One patient, a 64-year-old man, had a co-infection with *T. pallidum* and HSV-2. *H. ducreyi* was not detected by the reference method. Ten patients (27.8%) had ulcers due to other reasons (Table 4): 2/10 patients (20%) had chancriform pyoderma, 1/10 (10%) had candida balanitis and in 7/10 patients (70%) ulcers remained of unknown origin. Multiple ulcers were present in 11/36 patients (31%). Ulcers in the genital and oropharyngeal region were present in 2/19 *T. pallidum*-positive patients (11%) and in 1/10 patients (10%) with ulcers due to other causes. Ulcers in the genital, anal and oropharyngeal region were found in the *T. pallidum*/HSV-2 co-infected patient.

Comparison between TP-HD-HSV1/2 PCR and reference method. The TP-HD-HSV1/2 PCR was concordant in the 17 patients who were *T. pallidum* positive by the reference methods and

TABLE 2. Comparison of the new multiplex real-time PCR with the reference PCR in the retrospective study using samples from reference laboratories in the Netherlands and Switzerland

	Treponema pallidum[a]			Herpes simplex virus type 1[b]			Herpes simplex virus type 2[c]		
	Reference method			Reference method			Reference method		
New real-time PCR	Positive No. (%)	Negative No. (%)	Total No. (%)	Positive No. (%)	Negative No. (%)	Total No. (%)	Positive No. (%)	Negative No. (%)	Total No. (%)
Positive	20 (100)	0 (0)	20 (10)	24 (100)	2 (1)	26 (13)	21 (100)	3 (2)	24 (12)
Negative	0 (0)	173 (100)	173 (90)	0 (0)	167 (99)	167 (87)	0 (0)	169 (98)	169 (88)
Total	20 (100)	173 (100)	193 (100)	24 (100)	169 (100)	193 (100)	21 (100)	172 (100)	193 (100)

[a]McNemar test p 1.
[b]McNemar test p 0.48.
[c]McNemar test p 0.25.

TABLE 3. Sensitivity and specificity of the new real-time multiplex PCR compared with the reference methods in the retrospective and the prospective clinical studies

	Retrospective study		Prospective clinical study		Overall	
Pathogen	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
Treponema pallidum	100 (76–100)	100 (97–100)	100 (78–100)	100 (73–100)	100 (88–100)	100 (97–100)
Herpes simplex type 1	100 (80–100)	99 (96–100)	100 (28–100)	96 (79–99)	100 (82–100)	98 (96–100)
Herpes simplex type 2	100 (77–100)	98 (95–100)	100 (36–100)	100 (79–100)	100 (77–100)	98 (95–100)

TABLE 4. Characteristics of 36 patients with genital, anal or oropharyngeal ulcers in the prospective clinical study

	Treponema pallidum (*n* = 19)[a]	Herpes simplex (*n* = 8)[a]	Other cause (*n* = 10)	p value[b]
Age, mean years (SD)	42 (±12)	43 (±13)	40 (±16)	0.95
Gender, male No. (%)	19 (100)	7 (88)	10 (100)	0.22
Site of ulcer, No. (%)				
Genital region	17 (90)	8 (100)	10 (100)	0.71
Perianal region	1 (5)	1 (13)	0 (0)	0.46
Oral region	5 (26)	1 (13)	1 (10)	0.64
Incubation period				
Median (IQR), days	30 (17–46)	12 (4–48)	n.a.	0.13[c]
Data N/A, No. (%)	6 (32)	2 (25)	n.a.	
Duration of ulcers				
Median (IQR), days	13 (7–19)	10 (3–14)	5 (1–10)	0.06
Data N/A, No. (%)	1 (5)	0 (0)	0 (0)	
Ulcers per patient				
Median number (IQR)	1 (1–3)	1 (1–2)	1 (1–1)	0.23
Multiple ulcers, No. (%)	8 (42)	3 (38)	1 (10)	0.21
Regional lymphadenopathy, No. (%)	14 (74)	4 (50)	2 (20)	0.06
Syphilis serology				
VDRL				
No. positive/No. performed (%)	10/16 (63)	1/3 (33)	0/4 (0)	0.08
Median titre (IQR)	1:4 (1:0–1:32)	1:0 (1:0–1:64)	1:0 (N/A)	0.05
IgM ELISA				
No. positive/No. performed (%)	12/16 (75)	1/2 (50)	0/4 (0)	0.04[d]
Median titre (IQR)	2.14 (0.57–3.17)	N/A (N/A)	0.22 (0.15–0.27)	0.06
TPPA				
No. positive/No. performed (%)	18/18 (100)	2/8 (25)	2/10 (20)	<0.001[d,e]
Median titre (IQR)	1:1280 (1:1280–1:5120)	1:0 (1:0–1:320)	1:0 (1:0–1:0)	<0.001[d], 0.003[e]
Dark field microscopy				
No. positive/No. performed (%)	3/8 (38)	0/5 (0)	0/5 (0)	0.22
Conventional HSV PCR				
No. positive/No. performed (%)	1/13 (8)	7/8 (88)	0/6 (0)	0.002[e], 0.007[f]
Haemophilus ducreyi culture				
No. positive/No. performed (%)	0/13 (0)	0/6 (0)	0/10 (0)	1

ELISA, enzyme-linked immune sorbent assay; HSV, herpes simplex virus; IgM, immunoglobulin M; IQR, interquartile range; n.a., not applicable; N/A, not available; SD, standard deviation; TPPA, *Treponema pallidum* particle agglutination test; VDRL, Venereal Disease Research Laboratory test.
[a]One patient was co-infected with *T. pallidum* and HSV-2.
[b]If not otherwise indicated, p values result from the Kruskal–Wallis test, ANOVA or Fisher's exact test for comparison between all groups. If p <0.05, pairwise comparison with adjustment for multiple comparison was done, and only p <0.05 of pairwise comparison are shown.
[c]Wilcoxon rank sum test was performed due to two groups.
[d]*T. pallidum* vs. other cause. p value adjusted for groupwise multiple comparison.
[e]*T. pallidum* vs. herpes simplex. p value adjusted for groupwise multiple comparison.
[f]Herpes simplex vs. other cause. p value adjusted for groupwise multiple comparison.

was positive in two additional patients; in one neither dark field microscopy nor serology was carried out, and in the other serology was negative while dark field microscopy was not carried out. Discrepancy analysis confirmed a *T. pallidum* infection in both, in line with the clinical and anamnestic information, resulting in a 100% concordance between the TP-HD-HSV1/2 PCR and the resolved data. Of note, 5/19 (26%) of *T. pallidum* PCR-positive patients had a non-reactive and 1/19 (5%) a low-reactive Venereal Disease Research Laboratory test (VDRL) while *Treponema pallidum* particle agglutination test (TPPA) was positive. Of these, three patients had a positive IgM-ELISA that became negative after treatment, two patients had a negative IgM-ELISA but a reactive VDRL test at follow-up 8 and 9 days later, and one patient was positive in the discrepancy analysis.

The TP-HD-HSV1/2 PCR was concordantly positive for HSV-1 or HSV-2 in the seven patients who were diagnosed by the reference methods. Two additional samples were HSV-1 positive by the TP-HD-HSV1/2 PCR. Discrepancy analysis showed one to be false-positive; this patient had a candida balanitis. It is assumed that the sample was cross-contaminated during DNA extraction. The other was regarded as true-positive; this patient was clinically diagnosed with genital herpes 6 months before enrollment in our study. Despite a then negative conventional HSV PCR, this diagnosis was made based on the clinical picture, the recurrence of ulcers and the cessation of symptoms after initiation of a suppressive antiviral therapy. The current episode with three ulcers at one labium majorum emerged after interruption of the suppressive antiviral therapy and cleared after restart of this therapy, substantiating the correct positive result of the TP-HD-HSV1/2 PCR. *H. ducreyi* was not detected by the TP-HD-HSV1/2 PCR or the reference method in any specimens.

Overall sensitivity and specificity

The overall sensitivity and specificity of the TP-HD-HSV1/2 PCR was calculated to be 100% each for *T. pallidum*, 100% and 98% for HSV-1 and 100% and 98% for HSV-2 (Table 3). There was no significant difference in the performance of the TP-HD-HSV1/2 PCR compared with reference methods (Tables 2, 3 and 5). Sensitivity and specificity could not be calculated for *H. ducreyi*.

TABLE 5. Cross-tabulation of the results of the new multiplex real-time PCR compared with the reference methods (resolved data) in the clinical study[a]

	Treponema pallidum[b]			Herpes simplex type 1[b]			Herpes simplex type 2[b]		
	Reference method			Reference method			Reference method		
New real-time PCR	Positive No. (%)	Negative No. (%)	Total No. (%)	Positive No. (%)	Negative No. (%)	Total No. (%)	Positive No. (%)	Negative No. (%)	Total No. (%)
Positive	22[c] (100)	0 (0)	22 (56)	4 (100)[d]	1 (4)	5 (18)	5 (100)	0 (0)	5 (18)
Negative	0 (0)	17 (100)	17 (44)	0 (0)	23 (96)	23 (82)	0 (0)	23 (100)	23 (82)
Total	22 (100)	17 (100)	39 (100)	4 (100)	24 (100)	28 (100)	5 (100)	23 (100)	28 (100)

[a]All numbers in the table refer to number of samples investigated, not patients.
[b]McNemar test p 1.
[c]Two of these samples were negative in the *T. pallidum* reference standard but positive for *T. pallidum* in the discrepancy analysis.
[d]One of these samples was negative for herpes simplex type 1 in the reference standard and the discrepancy analysis, but medical history and disease course suggested a herpes simplex infection.

Discussion

We developed and evaluated the new TP-HD-HSV1/2 PCR for laboratory diagnosis of genital, anal and oropharyngeal ulcers. The primers and probes for *T. pallidum* and *H. ducreyi* were novel, and the performance in testing for *T. pallidum* was excellent (100% sensitivity and specificity). The primers specific for HSV-1/2 were developed and successfully tested by others on more than 3000 clinical specimens [21]. They found a sensitivity of >99.5% for both serotypes, which is in line with our results.

The TP-HD-HSV1/2 PCR was positive for *T. pallidum* in the samples of six patients with negative (n = 5) or equivocal (n = 1) VDRL. In three of these patients, the IgM-ELISA test was positive, corroborating the suggested higher sensitivity of a specific IgM test compared with the VDRL test in early syphilis [11,30]. However, the IgM test was equivocal in one of these patients and negative in two patients, which indicates that in very early syphilis molecular methods such as our TP-HD-HSV1/2 PCR may be superior to serology. The TP-HD-HSV1/2 PCR was also accurate in samples from oropharyngeal ulcers. These samples are not suitable for dark field microscopy. However, the high frequency of unprotected oral sex and the often underestimated rate of oral ulcers caused by sexually transmitted pathogens [31] substantiate the need for a PCR that reliably detects sexually transmitted pathogens also in oropharyngeal ulcers [25,32]. The detection of mixed infections by PCR in one patient of the present study as well as in earlier trials [33,34] supports the usefulness of PCR assays capable of a simultaneous and fast detection of *T. pallidum*, HSV-1/2 and *H. ducreyi* in a patient. Previously developed multiplex PCRs for these pathogens, including an assay capable of the simultaneous detection of seven different sexually-transmitted pathogens, are mostly conventional PCR assays, which need time-consuming subsequent electrophoresis to detect PCR products (for example [35–39]). Only a few multiplex real-time PCR assays have been developed [2,33,34], and only two have been tested for their applicability in routine clinical practice [33,34].

We sought to determine the rate of *T. pallidum*, HSV-1/2 and *H. ducreyi* in ulcers of our patients from Zurich, Switzerland, and Graz, Austria. The proportion of 53% of *T. pallidum*-positive patients in the present study appeared higher than the rates of ulcers caused by syphilis reported from other European areas, for example 2% in the UK [33], 3% in the Netherlands [3] or 35% in France [1]. Rates of reported cases of *T. pallidum* infections vary across European countries from <1 to 11/100 000 population in 2011, with an overall decrease since 2002 [40]. In Switzerland, which is not included in the above data from the European Centre for Disease Prevention and Control, the rate of reported *T. pallidum* infections was higher, with 12.4/100 000 in 2011 and an overall increase of 50% since 2006 [41]. This may have contributed to the high rate of *T. pallidum* infections in our patients. Another reason may be the higher male-to-female ratio of 35:1 found here, as compared with other European reports with a male-to-female ratio of 7:1 [1] or 1.5:1 [3]; as ulcers inside the vagina often remain undetected, syphilis is reported almost four times more often in men than in women [40]. This skewed gender ratio might be due to the exclusive recruitment of our patients in centres for dermatology or infectious diseases, whereas women with genital ulcers usually see their gynaecologist.

HSV-1/2 was detected in 22% of our patients, and the rate of HSV among ulcer patients reported by previous PCR studies varied between 12.5% and 63% [33–37]. In previous decades, genital herpes was primarily caused by HSV-2, but during the current century, the proportion of HSV-1 as a causative agent of genital herpes has increased in many developed countries [42]. While recent studies from Africa and South America report an HSV-2:HSV-1 ratio in the range of 10:1–20:1 in

favour of HSV-2 [34,36,37], one from the UK found a 1:1 ratio [33] as we did.

The absence of *H. ducreyi* in our samples is in line with European surveillance data from the last decade, reporting *H. ducreyi* in 0.9–3% of ulcer patients from the Netherlands [3] or France [1]. This low rate of chancroid in Europe observed over the last 15 years is supposedly due to shifts in social and public health conditions, such as improved hygiene [43]. However, the development and availability of an *H. ducreyi*-specific real-time PCR in Europe is useful for several reasons. First, *H. ducreyi* is prevalent in many geographical regions; for example, it is detected in up to 51% of patients with genital ulcers in Sub-Saharan Africa [34,36,44] and travellers or immigrants from endemic regions seen in industrialized countries might suffer from chancroid [45,46]. Second, diagnostic tools other than PCR, such as microscopy and cultivation, are not suitable for routine clinical practice [15]. These insensitive techniques would hamper the diagnosis of rare cases. To address these issues in the United States, the Centers for Disease Control and Prevention have recently developed a PCR assay for *H. ducreyi* [2].

A limitation of the present study is that the HIV status of patients was not known, which is due to restrictions of the study protocol approved by the ethics committees in Switzerland and Austria. Another limitation of our TP-HD-HSV1/2 PCR is that other rare causes of genital, anal or oropharyngeal ulcers (e.g. *Chlamydia trachomatis* genotypes L1-L3, cytomegalovirus and Epstein-Barr virus) are not included.

In conclusion, the TP-HD-HSV1/2 PCR proved to be sensitive and specific for the detection of *T. pallidum* and HSV-1/2 in patients with genital, anal or oropharyngeal ulcers. This PCR is applicable in routine clinical practice and results are available within 24 h after sampling. More patients with suspected early *T. pallidum* infections but negative VDRL test should be tested with the TP-HD-HSV1/2 PCR to confirm its supposed SUPERIOR sensitivity compared with serology in these types of specimens.

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Transparency Declaration

The authors declare no conflicts of interest.

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