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Use of a Multiplex PCR Assay To Assess the Presence of *Treponema pallidum* in Mucocutaneous Ulcerations in Patients with Suspected Syphilis

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ABSTRACT We evaluated the utility of the commercial Allplex genital ulcer real-time PCR multiplex assay for detecting *Treponema pallidum*, herpes simplex virus 1 (HSV-1) and 2 (HSV-2), and *Chlamydia trachomatis* serovar L (lymphogranuloma venereum [LGV]) DNA in mucosal and genital ulcers in the context of suspected syphilis. In total, 374 documented genital and mucosal ulcers from patients with and without syphilis presenting at several sexually transmitted infection (STI) centers in France from October 2010 to December 2016 were analyzed at the National Reference Center (CNR) for Bacterial STIs at Cochin Hospital in Paris. *T. pallidum* subsp. *pallidum* detection results were compared with the final diagnosis based on a combination of clinical examination, serological results, and in-house nested PCR (nPCR). Detections of HSV and LGV were validated against reference methods. We found that 44.6% of the 374 samples tested were positive for *T. pallidum* subsp. *pallidum*, 21% for HSV, and 0.8% for LGV. No positive results were obtained for 30.7% of samples, and 4.8% presented coinfections. For *T. pallidum* subsp. *pallidum* detection, the overall sensitivity was 80% (95% confidence interval [CI], 76.1 to 84.1%), specificity was 98.8% (95% CI, 97.7 to 99.9%), positive predictive value was 98.8% (95% CI, 97.7 to 99.9%) and negative predictive value was 80.2% (95% CI, 76.2 to 84.2%), with a rate of concordance with the reference method of 92.5% ($k = 0.85$). This PCR multiplex assay is suitable for *T. pallidum* subsp. *pallidum* detection in routine use and facilitates the simultaneous rapid detection of a broad panel of pathogens relevant in a context of suspected syphilis lesions.

KEYWORDS detection, multiplex PCR, *Treponema pallidum*, syphilis

Syphilis is a multistage disease caused by the spirochete *Treponema pallidum* subsp. *pallidum*, which infects about 5.6 million people worldwide per year (1). Syphilis is mostly sexually transmitted and is characterized by several mucosal (genital, anal, and oral) and cutaneous lesions containing viable *Treponema* bacteria, mostly in HIV-infected patients and men who have sex with men (MSM) (2). Syphilis is a slowly

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progressing chronic disease in which the host-pathogen relationship is continually changing. Indeed, it is customary to group the different phases of syphilis into two categories (early syphilis and late syphilis) according to similarities in management, the degree of contagiousness, and neurological risk. Early syphilis covers the primary, secondary, and early latent forms of less than one year's duration, all of which are highly contagious, and possible early neurosyphilis, which is now the most common form of neurosyphilis. Late syphilis covers late latent syphilis of more than one year's duration and tertiary syphilis with skin, cardiovascular, and neurological complications, which are observed very rarely, despite the reemergence of syphilis over the last two decades or so (3).

Dark-field microscopy (DFM) examination is no longer used for the direct detection of *T. pallidum* subsp. *pallidum* (4), and no bacteriological tests has been developed, despite recent improvements in *T. pallidum* subsp. *pallidum* culture techniques (5). Syphilis diagnosis is thus currently based solely on indirect serological treponemal tests (TT) and nontreponemal tests (TNT) (6–14). Confirmation of the diagnosis of syphilis is based principally on a combination of serological tests, clinical examination, and PCR testing, with many laboratories now using in-house PCR techniques (15).

In this study, we evaluated a new commercial real-time multiplex PCR assay for simultaneous detection of the genomes of *Treponema pallidum*, herpes simplex viruses 1 (HSV-1) and 2 (HSV-2), and *Chlamydia trachomatis* serovar L (the causal agent of lymphogranuloma venereum [LGV]). DNA was extracted from genital and mucosal swabs collected from patients with documented mucocutaneous lesions in whom syphilis was suspected. We tested for the presence of *T. pallidum* subsp. *pallidum*, HSV, and LGV in lesions in patients with suspected syphilis and evaluated the performance of this assay for *T. pallidum* subsp. *pallidum* detection, comparing the results obtained with those for our routine nested PCR (nPCR) assay and with the final diagnosis.

MATERIALS AND METHODS

Patients. Patients presenting at six STI centers in France for suspected syphilis were enrolled in the study, after informed consent had been obtained, from October 2010 to December 2016.

Study population. In total, 374 patients were enrolled, and one swab per patient was obtained from genital, oral, buccal, and cutaneous ulcers clinically and serologically documented as part of the GENOSYPH study (CPP S.C. 3005, CNIL no. 1208504) initiated by the National Reference Center for Bacterial STIs in 2010. The anonymized samples, from STI centers in Paris (at the Cochin and Saint-Louis Hospitals), Metz-Thionville, Nancy, Valenciennes, Marseille, and Aix-en-Provence, were obtained from patients consulting for STIs with suspected recent syphilis. Recent syphilis was defined as (i) primary syphilis with one or more syphilitic ulcers (chancres), with evidence of *T. pallidum* subsp. *pallidum* in samples on DFM examination or in validated routine genomic amplification assays (12), and positive serological results in NTT (Venereal Disease Research Laboratory [VDRL] or rapid plasma reagin [RPR]) or TT (*Treponema pallidum* hemagglutination [TPHA] or enzyme-linked immunosorbent assay [ELISA]) or (ii) secondary syphilis in patients with skin and/or mucosal lesions associated with positive NTT and TT results. The final diagnosis was based on a combination of clinical examination, serological results, and in-house nested PCR (12). The gold standard was the final diagnosis reached by consensus concerning the clinical status of each patient, reached by discussion at a meeting of the physicians participating in the study.

Sample processing. Mucosal ulcers or erosions and skin lesions were gently scraped with a scalpel to obtain a serous exudate, which was collected with a swab, placed in 1 ml of phosphate-buffered saline (PBS) (1.5 mM KH_2PO_4, 2.7 mM $Na_2HPO_4{\cdot}7H_2O$, and 0.15 M NaCl [pH 7.4]) and stored at −20°C until DNA extraction. All samples were cryptically labeled to conceal the identity of the patient and to ensure that assessments were performed in a blind manner, without knowledge of the final result, so as not to influence the reading.

Serological tests. Serum samples from all patients included in this study were routinely tested with the RPR test (ASI RPR card test; Arlington Scientific, Springville, UT) and by ELISA (Architect Syphilis TP system; Abbott, Abott Park, IL), according to the kit manufacturers' instructions. We tested for antibodies against HIV-1 and HIV-2 by routine automated ELISA (Genscreen Ultra HIV Ag-Ab; Bio-Rad, Marne-la-Coquette, France), according to the manufacturer's instructions.

DNA extraction. We performed DNA extraction on each of the swab exudates with the InnuPrep Blood DNA-IPC16 minikit assay on an InnuPure C16 instrument (Analytikjena, Jena, Germany), in accordance with the manufacturer's instructions. Briefly, we introduced 200 μl of sample into the reagent plate or strip of the prefilled cartridges, in the presence of proteinase K. This method is based on enzymatic lysis at high temperature and separation with nucleic acid-binding magnetic particles. All swab exudates were thawed at 4°C for 18 h before extraction. Samples and extraction kit materials were left at room temperature for 15 min before the extraction procedure. Extractions were performed on 23 sets of 16 swabs and one set of six swabs. DNA was eluted in 50 μl of elution buffer and was stored at −20°C.

Amplification analysis. Extracted DNA was tested with a validated in-house nested PCR assay (nPCR) for detection of the *tpp47* gene of *T. pallidum* subsp. *pallidum* with the following primers: Tpe, 5′-CGTGCGAAGGTAGTGGCCCAGTAG-3′; Tps, 5′-TTCGATGCAGTTTCTCGCGCAACC-3′; KO5, 5′-AGGCTGACTTTGATTGCGAACGGG-3′; and KO3B, 5′-GACGCGAGCTACACCAATCTGATG-3′, as previously described (12). All PCRs were performed in a ProFlex PCR system (Applied Biosystems, Foster City, CA). Briefly, the first PCR assay was carried out in a final volume of 25 μl, containing 10 μl extracted template DNA. Positive and negative controls were run in parallel. The second PCR assay was carried out in a final volume of 25 μl, including 5 μl of product from the first PCR used as a template. Amplicons were analyzed by electrophoresis in a 2% agarose gel under UV illumination, in the presence of ethidium bromide. Multiplex real-time PCR (mPCR) was performed with the PCR multiplex Allplex genital ulcer assay (Seegene-Eurobio, Les Ulis, France), which can simultaneously detect DNA from HSV-1, HSV-2, *Haemophilus ducreyi* (HD), *Cytomegalovirus* (CMV), *Chlamydia trachomatis* serovar L, *T. pallidum* subsp. *pallidum*, and varicella zoster virus (VZV). Each kit contains 50 tests. Multiplex PCR was performed in a CFX96 real-time PCR system (Bio-Rad). Briefly, the assay was carried out in a final volume of 20 μl containing 5 μl of extracted template DNA, in accordance with the manufacturer's instructions. All positive detections of HSV-1 and HSV-2 were validated by real-time PCR (Artus HSV-1/2 PCR kit; Qiagen France, Les Ulis, France) at the National Reference Center (CNR) for Herpesviruses, and all detections of LGV were validated by an in-house real-time PCR assay developed at the CNR for Bacterial STIs specializing in chlamydial infections (16).

Statistical analysis. We assessed test performance by calculating sensitivity (Se), specificity (Sp), and positive and negative predictive values (PPV and NPV) by standard methods. We used χ^2 tests and Fisher's exact tests to investigate the associations between positive PCR detections and serological status for HIV. Associations between continuous variables were investigated with nonparametric tests (Mann-Whitney U tests). *P* values of <0.05 in two-tailed tests were considered significant. McNemar and kappa indices were calculated to compare the proportion of positive samples and the degree of agreement between the two methods, respectively.

RESULTS

Characteristics of the study population. We included 374 patients (344 men and 30 women) with a median age of 37.85 years (95% confidence interval [CI], 36.53 to 39.18) in this study. Most were MSM (61.5%), and 22.1% had HIV infection. The study population consisted of 134 patients with primary syphilis (65%), 72 patients with secondary syphilis (35%), and 168 control patients with mucosal ulcers who were not diagnosed with syphilis. The lesions were located in the genital area in 249 cases (66.5%), at anal sites in 66 cases (17.6%), at oral sites in 42 cases (11.2%), and were found on the skin in 17 cases (4.5%) (Table 1).

Detection of the *T. pallidum* subsp. *pallidum* genome. With the mPCR assay, we were able to detect the *T. pallidum* subsp. *pallidum* genome with an overall sensitivity of 80% (95% CI, 76.1 to 84.1%) and an overall specificity of 98.8% (95% CI, 97.7 to 99.9%). The mean of threshold cycle (C_T) was 27.65 (95% CI, 27.12 to 28.19%). The positive and negative predictive values (PPV and NPV) were 98.8% (95% CI, 97.7 to 99.9%) and 80.2% (95% CI, 76.2 to 84.2%), respectively. All of the samples analyzed ($n = 374$) were validated by detection of the internal control, with a mean C_T value of 25.98 (95% CI, 25.61 to 26.35%). In comparison, our routine nPCR assay had an overall sensitivity of 84% (95% CI, 80.3 to 87.7%) and a specificity of 100%, with a PPV of 100% and an NPV of 83.6% (95% CI, 79.8 to 87.3%) (Table 2). Sensitivity depended on lesion location. The two assays had similar sensitivities for lesions at oral sites (92%). However, for genital, anal, and cutaneous lesions, sensitivity was higher with the nPCR assay (Table 2). A McNemar test on the proportion of positive samples showed no significant difference ($P = 0.345$) between the two tests. Their results were concordant in 92.5% of cases, with a kappa index of 0.85 (95% CI, 79.5 to 90.3%) (Table 3). We also compared the performance of the two techniques as a function of syphilis status. For primary syphilis, we obtained a sensitivity of 80.6% (95% CI, 73.1 to 86.4%), with a PPV of 100% (95% CI, 96.6 to 100%) for mPCR and a sensitivity of 83% (95% CI, 75.6 to 88.2%) with a PPV of 100% (95% CI, 96.6 to 100%) for nPCR. No significant difference in positive detection rates was found between the two tests ($P = 0.345$). Moreover, the concordance rate was at 88.8%, with a kappa index of 0.63 (95% CI, 45.3 to 79.8%). Interestingly, the sensitivity values for *T. pallidum* subsp. *pallidum* DNA detection in genital and buccal lesions were very similar between the two assays (82% for mPCR and 85% for nPCR), whereas in anal and cutaneous lesions, the sensitivity values were higher for nPCR (81% and 100%, respectively) than for mPCR (71% and 75%, respectively) (Table 2). For

TABLE 1 Population characteristics

Characteristic	Patients at syphilis stage[b]: Primary	Secondary	Patients without syphilis[b]
Total no. of patients	134	72	168
Population			
Men	132 (98)	71 (97)	141 (84)
Women	2 (1.5)	1 (1)	27 (16)
Age (yrs)			
Median	38.8	41.3	35.9
Range	21–71	19–69	18–71
Sexual orientation[a]			
MSM	106 (79)	55 (76)	69 (41)
MSW	21 (16)	9 (12.5)	90 (53)
NS	7 (5)	8 (11)	9 (5)
Type of clinical lesion			
Genital	96 (72)	34 (47)	119 (71)
Anal	21 (16)	16 (22)	29 (17)
Buccal	13 (10)	13 (18)	16 (9.5)
Cutaneous	4 (3)	9 (12.5)	4 (2.4)
Infection status			
HIV positive	37 (28)	30 (42)	16 (9.5)

[a]MSM, men who have sex with men; MSW, men who have sex with women; NS, not specified.
[b]Given as *n* (%) except for age (median and range in yrs).

secondary syphilis, the sensitivity was 79.2% (95% CI, 68.4 to 86.9%), with a PPV of 100% (95% CI, 93.7 to 100%) for mPCR, whereas the sensitivity was 86.1% (95% CI, 76.3 to 92.3%), with a PPV of 100% (95% CI, 94.2 to 100%) for nPCR. A McNemar test revealed that there was no significant difference ($P = 0.228$) in positive detection rates between the two tests. A concordance rate of 84.7% (95% CI, 21.0 to 73.4%) was found, with a kappa index of 0.47 (95% CI, 21.0 to 73.4%) (Table 3).

Detection of the *T. pallidum* subsp. *pallidum* genome and patient HIV status. For the two assays, *T. pallidum* subsp. *pallidum* DNA detection rates were higher in HIV-negative patients than in HIV-positive patients (sensitivity of mPCR, 89% [95% CI,

TABLE 2 Comparison of *T. pallidum* detection with the mPCR and nPCR assays

Diagnosis	Clinical material: Nature	*n*	*T. pallidum* molecular detection[c]: mPCR[a] Pos (*n* [%])	Se (%)	Sp (%)	nPCR[b] Pos (*n* [%])	Se (%)	Sp (%)
Primary syphilis (*n* = 134)	Genital	96	79 (82)	82		79 (82)	82	
	Anal	21	15 (71)	71		17 (81)	81	
	Buccal	13	11 (85)	85		11 (85	85	
	Cutaneous	4	3 (75)	75		4 (100)	100	
Secondary syphilis (*n* = 72)	Genital	34	27 (79)	79		30 (88)	88	
	Anal	16	11 (69)	69		12 (75)	75	
	Buccal	13	12 (92)	92		12 (92)	92	
	Cutaneous	9	7 (78)	78		8 (89)	89	
No syphilis (*n* = 168)	Genital	119	1 (0.8)		99.2	0		100
	Anal	29	1 (3.4)		96.6	0		100
	Buccal	16	0		100	0		100
	Cutaneous	4	0		100	0		100

[a]Overall, Se = 80%, Sp = 98.8%, positive predictive value (PPV) = 98.8%, and negative predictive value (NPV) = 80.2%.
[b]Overall, Se = 84%, Sp = 100%, PPV = 100%, and NPV = 83.6%.
[c]Pos, positive; Se, sensitivity; Sp, specificity.

TABLE 3 Discrepancies in *T. pallidum* genome detection between mPCR and nPCR assays

		mPCR[d]					
		Overall[a]		Primary syphilis[b]		Secondary syphilis[c]	
Syphilis stage	nPCR assay result (*n*)	Pos (*n*)	Neg (*n*)	Pos (*n*)	Neg (*n*)	Pos (*n*)	Neg (*n*)
All stages	Pos	156	17				
	Neg	11	190				
Primary syphilis	Pos			102	9		
	Neg			6	17		
Secondary syphilis	Pos					54	8
	Neg					3	7

[a]"Overall" corresponds to the samples from patients with syphilis and patients without syphilis ($n = 374$); 92.5% agreement between mPCR and nPCR; kappa index of 0.85 (95% CI, 0.79 to 0.90).
[b]Primary syphilis ($n = 134$); 88.8% agreement between mPCR and nPCR; kappa index of 0.63 (95% CI, 0.45 to 0.80).
[c]Secondary syphilis ($n = 72$); 84.7% agreement between mPCR and nPCR; kappa index of 0.47 (95% CI, 0.21 to 0.73).
[d]Pos, positive; Neg, negative.

81.7 to 93.0%] versus 69% [95% CI, 56.8 to 78.5%] [$P = 0.008$], respectively; sensitivity of nPCR, 91% [95%CI, 84.6 to 94.9%] versus 75% [95% CI, 63.1 to 83.5%] [$P = 0.002$], respectively) (Table 4).

Genome detection in the mPCR assay. We did not investigate *H. ducreyi*, VZV, and CMV further, even though they were components of the panel, because of their very low detection rates and/or the lack of a predetermined adjudication method.

For primary syphilis, 82% (79/96) of the 96 genital samples were positive for *T. pallidum* subsp. *pallidum* (Table 2), 6% (6/96) were positive for HSV-1, and 1% (1/96) each were positive for HSV-2 and LGV. Four of the six genital samples were coinfected with HSV-1 (Tables 5 and 6). We found that 71% (15/21) of the anal samples were positive for *T. pallidum* subsp. *pallidum* (Table 2), 14% (3/21) for HSV-1, 9% (2/21) for HSV-2, and 5% (1/21) for LGV. Three samples were coinfected with *T. pallidum* subsp. *pallidum*, HSV-1, and HSV-2. One sample was coinfected with HSV-1 and HSV-2 (Table 6). Among the buccal samples, 85% (11/13) were positive for *T. pallidum* subsp. *pallidum* (Table 2), and only 8% (1/13) were positive for HSV-1 (Table 5). For the four cutaneous samples, 75% (3/4) were positive for *T. pallidum* subsp. *pallidum* only. Overall, 81% (108/134) of the lesions identified in patients with primary syphilis were positive for *T. pallidum* subsp. *pallidum* DNA (Table 2).

For secondary syphilis, 79% (27/34) of the genital samples were positive for *T. pallidum* subsp. *pallidum* (Table 2) and 9% (3/34) for HSV-1. One sample was coinfected with HSV-1 (Tables 5 and 6). We found that 69% (11/16) of the anal samples tested positive for *T. pallidum* subsp. *pallidum* (Table 2) and 6% (1/16) for HSV-1 and HSV-2. One sample was coinfected with HSV-2 (Table 6). For the buccal samples, 92% (12/13) were positive for *T. pallidum* subsp. *pallidum* (Table 2) and 23% (3/13) for HSV-1. Three samples were coinfected with *T. pallidum* subsp. *pallidum* and HSV-1 (Tables 5 and 6).

TABLE 4 Detection of the *T. pallidum* genome, by HIV status

	HIV status[a]								
	Positive				Negative				
PCR assay	Total (*n*)	Pos (*n* [%])	Se (%)	Sp (%)	Total (*n*)	Pos (*n* [%])	Se (%)	Sp (%)	*P*
mPCR	83	47 (56.6)	69	94	271	109 (40.2)	89	99	0.008[d]
nPCR	83	50 (60.2)	75	100	271	111 (40.9)	91	100	0.002[e]

[a]Pos, positive; Se, sensitivity; Sp, specificity.
[d]$\chi^2_1(1, N = 354) = 9.5$.
[e]$\chi^2_1(1, N = 354) = 6.9$.

TABLE 5 Multiplex assay detection in mucocutaneous lesions from patients with suspected syphilis

Diagnosis	Clinical material		Multiplex molecular detection of[a]:		
	Nature	Total (*n*)	HSV-1 Pos (*n* [%])	HSV-2 Pos	LGV Pos (*n* [%])
Primary syphilis	Genital	96	6 (6)	1 (1)	1 (1)
	Anal	21	3 (14)	2 (9)	1 (5)
	Buccal	13	1 (8)	0	0
	Cutaneous	4	0	0	0
Secondary syphilis	Genital	34	3 (9)	0	0
	Anal	16	1 (6)	1 (6)	0
	Buccal	13	3 (23)	0	0
	Cutaneous	9	0	0	0
No syphilis	Genital	119	16 (13)	26 (22)	0
	Anal	29	7 (24)	7 (24)	1 (3)
	Buccal	16	1 (6)	0	0
	Cutaneous	4	0	0	0

[a]HSV-1, herpes simplex virus 1; HSV-2, herpes simplex virus 2; LGV, lymphogranuloma venereum, *Chlamydia trachomatis* serovar L; Pos: positive.

Overall, 79.2% (57/72) of the lesions identified in patients with secondary syphilis were positive for *T. pallidum* subsp. *pallidum* DNA (Table 2).

For all lesions in nonsyphilis patients, 34% (57/168) were positive for HSV, 14% (24/168) for HSV-1 and 20% (33/168) for HSV-2, and 0.6% (1/168) for LGV. HSV DNA was mostly detected in genital lesions, 13% (16/119) and 22% (26/119) of which were positive for HSV-1 and HSV-2, respectively (Table 5). HSV-1 and HSV-2 were detected in 48% (14/29) of anal lesions, whereas only 6% of buccal lesions tested positive for HSV-1. LGV was detected in 3% (1/29) of anal lesions. However, with the mPCR assay, *T. pallidum* subsp. *pallidum* was detected in 0.8% (1/119) of genital ulcers and 3% (1/29) of anal lesions (Table 2).

DISCUSSION

Serological tests are important for syphilis diagnosis, but the direct detection of *T. pallidum* subsp. *pallidum* by DFM and/or PCR is also potentially valuable. There is, therefore, a need to develop rapid and specific methods for amplifying the genome of *T. pallidum*, for detecting the presence of the spirochete in samples. Several PCR

TABLE 6 Coinfections detected by the multiplex assay

Diagnosis	Nature of clinical material	Sampling date	Multiplex molecular detection[a]				Syphilis serology[b]		HIV status[c]
			TPA	HSV-1	HSV-2	LGV	NTT	TT	
Primary syphilis	Genital	27 February 2013	Pos	Pos			32	Pos	Neg
		5 March 2013	Pos	Pos			4	Pos	nr
		28 March 2013	Pos	Pos			16	Pos	Neg
		7 July 2016	Pos	Pos			8	Pos	Neg
	Anal	25 February 2011	Pos	Pos			256	Pos	Pos
		17 September 2012	Pos		Pos		Neg	Pos	nr
		24 January 2013	Pos	Pos	Pos		32	Pos	Neg
Secondary syphilis	Genital	18 February 2013	Pos	Pos			32	Pos	Neg
	Anal	6 May 2013	Pos		Pos		8	Pos	Pos
	Buccal	13 February 2013	Pos	Pos			32	Pos	Pos
		14 February 2013	Pos	Pos			16	Pos	Pos
		22 April 2016	Pos	Pos			32	Pos	nr

[a]TPA, *T. pallidum*; HSV-1, herpes simplex virus 1; HSV-2, herpes simplex virus type 2; LGV, lymphogranuloma venereum, *Chlamydia trachomatis* serovar L.
[b]NTT, nontreponemal test (VDRL or RPR); TT, treponemal test (TPHA, chemiluminescent microparticle immunoassay [CMIA], or enzyme-linked immunosorbent assay [ELISA]).
[c]HIV status was determined by ELISA. Neg, HIV negative; Pos, HIV positive; nr, not recorded.

methods have been developed for use on various clinical specimens, such as serum, cerebrospinal fluid, amniotic fluid, placenta, blood, swabs from cutaneous syphilis lesions, bone, aorta, neurons, urine, and gastric fluid. Evaluations of PCR detection performances on large populations of patients with suspected syphilis have reported sensitivities ranging from 57% to 97% and specificities of 95% to 100%, and have shown that PCR detection can replace DFM in situations in which DFM is not available (8–10, 12, 13, 17–21). In-house multiplex real-time PCR assays capable of detecting several sexually transmitted microorganisms simultaneously in mucosal and cutaneous lesions have been evaluated in various populations (22). The previously described multiplex PCR assays can detect up to 11 different sexually transmitted microorganisms in addition to *T. pallidum* subsp. *pallidum* (23–29). In this study, we found that 44.6% of the samples tested were positive for *T. pallidum* subsp. *pallidum* with the mPCR assay. This result was compared with the gold standard, namely, final diagnosis of syphilis on the basis of clinical examination, serological tests, and in-house nPCR detection (12). In this study, the sensitivity and specificity of the nPCR assay were 84% and 100%, consistent with our previous findings of 82% and 95%, respectively (12). For the mPCR assay, sensitivity and specificity were 80% and 99%, respectively, and the agreement between the two assays was very good (92.5%). However, when performance as a function of syphilis status was considered, detection sensitivity was similar for the two assays for primary syphilis, but slightly higher for the in-house nPCR assay for secondary syphilis (12). The performance of this commercial mPCR assay for *T. pallidum* subsp. *pallidum* detection was similar to that of other assays. Previous studies describing similar in-house mPCR assays reported sensitivities of 45% to 100% and a specificity of 100% for *T. pallidum* subsp. *pallidum* detection relative to syphilis serology or final diagnosis (17, 24). A previous study suggested that detection sensitivity differed between anatomic sites. However, the interpretation of these results was limited by the small number of samples of anal and buccal origin (12). We show here that the sensitivity of *T. pallidum* subsp. *pallidum* detection was of the same order of magnitude for swabs of genital, anal, oral, and cutaneous origin. It has been known for several years that syphilis can contribute to HIV transmission and that, conversely, the immunosuppression induced by HIV can interfere with syphilis development. In this study, we found that *T. pallidum* subsp. *pallidum* detection rates were lower in HIV-positive patients. This result suggests that *T. pallidum* subsp. *pallidum* genomic load may be lower in HIV-positive patients, but this result requires validation in a larger patient population that includes many more HIV-positive patients in particular.

False-negative results were obtained for 50 patients, 33 with the nPCR and 41 with the mPCR. Interestingly, 24 patients had false-negative results in both the mPCR and nPCR assays. Five of these 24 false-negative samples were positive for HSV-1, one sample was positive for HSV-2, and one sample was positive for LGV. In contrast, 26 false-negative results were obtained for one PCR assay only. Four samples were positive for HSV-1, and two samples were false-negative for *T. pallidum* subsp. *pallidum* in the two PCR assays. One patient was positive for LGV and false-negative for *T. pallidum* subsp. *pallidum* in the mPCR assay only. For mPCR, the presence of PCR inhibitors that might interfere with the reaction was ruled out, because all samples presented a correct amplification signal for the internal control. As both assays are based on PCR, they can detect very small amounts of *T. pallidum* subsp. *pallidum* DNA, and the lack of detection may reflect the absence of *T. pallidum* subsp. *pallidum* or its presence at extremely low levels in the sample. Furthermore, some sampling may have been inadequate, particularly for secondary syphilis lesions, in which *T. pallidum* subsp. *pallidum* is located below the epidermis, necessitating careful scraping to ensure that the bacteria are correctly collected. It is also possible that patients had applied antiseptics to the lesions. Finally, we cannot exclude the possibility of degradation of the *T. pallidum* subsp. *pallidum* DNA during sampling.

False-positive results were obtained with the mPCR for two samples presenting C_T values of 36.52 and 27.92. Both samples yielded negative serological results for syphilis. One of the patients was diagnosed with vulvar candidiasis, and the other was

diagnosed with nonspecific anal ulceration. Interestingly, the mPCR detected HSV-2 in both samples, as confirmed by the CNR for Herpesviruses. Cross-contamination between specimens could be ruled out, because specimens were always handled separately, and cross-contamination would have led to a positive result being obtained for the nPCR assay too. The analytical process took place in three different rooms dedicated to PCR analysis. We cannot, therefore, exclude the possibility of nonspecific detection by the mPCR assay or contamination during the setting up of the assay, given that the DNA templates were added manually to a 96-well plate for the mPCR.

In this study, 21% of the samples tested positive for HSV-1 and HSV-2, with HSV-1 infections detected in 11% of patients. HSV infections were mostly detected in genital ulcers (14%) and were particularly frequent in patients without syphilis (11%). Thus, the HSV infection rate was high in our population, consistent with the findings of a previous study based on comparable clinical samples (24, 30).

In total, we detected 12 coinfections within syphilis lesions with the mPCR assay. We found that 86% of primary syphilis ulcers were coinfected with HSV-1. Interestingly, a similarly high rate of *T. pallidum* subsp. *pallidum*-HSV coinfection was reported in a population of HIV-positive MSM (30). One ulcer was coinfected with HSV-2, and another was found to be simultaneously coinfected with three different microorganisms, *T. pallidum* subsp. *pallidum*, HSV-1, and HSV-2. For secondary syphilis, we found that 80% of the coinfections involved HSV-1, which was detected predominantly in buccal lesions. One anal lesion was coinfected with HSV-2.

Syphilis rates worldwide have now reached their highest levels in almost 3 decades, and new approaches for the control of this disease, including diagnostic tests yielding more rapid results, are urgently required. The possibility of detecting several microorganisms in a single sample is an attractive one, as syphilis and HSV have identical epidemiologic profiles, and coinfections are observed in some individuals. Further studies will be required to evaluate the performance of this test for detecting HSV, LGV, CMV, VZV, and *H. ducreyi*.

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