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Evaluation of a laboratory developed multiplex real-time PCR assay for diagnosis of syphilis, herpes, and chancroid genital ulcers in four public health laboratories in the United States

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Abstract

Objectives—To evaluate the field performance of a multiplex PCR (M-PCR) assay for detection of herpes simplex virus (HSV)- 1 and HSV-2, *Treponema pallidum (T. pallidum),* and *Haemophilus ducreyi (H. ducreyi)* in genital ulcer disease (GUD) specimens.

Methods—GUD M-PCR was performed on 186 remnant specimens, previously collected for HSV testing, by four public health laboratories (PHLs) and the Laboratory Reference and Research Branch (LRRB) at Centers for Disease Control and Prevention (CDC). Results from the PHLs were compared to those of LRRB, which served as the reference testing method, and percentage agreement was calculated.

Results—HSV was detected in 31/52 (59.6%), 20/40 (50%), 43/44 (97.7%), and 19/50 (38.0%) specimens from PHL1-4, respectively. The overall percent agreement between PHLs and LRRB for HSV testing was 94-100% with a kappa value of 0.922, which demonstrates high agreement. *T.*

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pallidum was identified in 7/51 (13.7%) specimens from PHL1 with 94.1% agreement and in 2/40 (5.0%) specimens from PHL2 with 100% agreement. LRRB identified three additional *T. pallidum* positive specimens from PHL1. The kappa value (0.849) for *T. pallidum* testing suggests good agreement. Consistent with the LRRB results, no *T. pallidum* was detected in specimens from PHL3 and PHL4, and *H. ducreyi* was not detected at any of the study sites.

Conclusions—The GUD M-PCR assay performed well in four independent PHLs and 12 suspected syphilis cases were identified in this study. The M-PCR assay could provide improved diagnostic options for GUD infections in state and local public health laboratories.

Introduction

In the United States (US), *T. pallidum* and HSV are the predominant causes of genital ulcer disease (GUD).[1] In 2019, there are 129,813 cases of all stages of syphilis including 38,992 cases of primary and secondary (P&S) syphilis. The rate of P&S syphilis has increased 11.2% since 2000-2001.[2] The seroprevalence of HSV-2 in US was 12.1% in 2015-16, and genital HSV-1 infections are on the rise among young adults.[3] The prevalence of chancroid has declined in the US since 1987 with eight reported cases in 2019.[2]

Several studies have identified GUD as an important risk factor for the transmission of human immunodeficiency virus (HIV).[4] Therefore, early diagnosis and treatment of GUD may contribute to reducing HIV incidence worldwide. Currently, syphilis diagnosis relies primarily on the use of both treponemal and nontreponemal serological assays. However, up to 46% of patients can be seronegative in primary syphilis when genital ulcers are present.[5] Direct detection of *T. pallidum* by darkfield microscopy is useful for moist lesions of P&S syphilis, but the test is not widely available in the US and relies on microscopists maintaining proficiency in the test. Nucleic acid amplification tests (NAATs) have been shown to be useful for the detection of *T. pallidum* in moist lesions of primary syphilis with sensitivities ranging from 72% to 95% depending on the assay target.[5] Currently there are at least 13 FDA-cleared molecular assays for the detection of HSV-1 and HSV-2, but FDA-cleared NAATs for *T. pallidum* are still not available.[6]

Syphilis and herpes lesions may be difficult to distinguish clinically, while M-PCR allows simultaneous detection of multiple pathogens in the same sample. The analytical sensitivity of the GUD M-PCR assay developed by the LRRB is approximately 10–100 copies per reaction for the three DNA targets with a PCR efficiency ranging from 90 to 110%.[7] This assay has been validated in compliance with the Clinical Laboratory Improvement Amendments (CLIA) for use at LRRB for the diagnosis of GUD since 2010. In this study, we evaluated the field performance of this assay in state and local PHLs.

Methods:

Study design and specimen collection

Selection of the four study sites and implementation of the GUD M-PCR were coordinated by LRRB and the Association of Public Health Laboratories (APHL). The study was approved by CDC (Project Determination #7155) and the Henry Ford Health System IRB in Michigan (IRB#00008660). This project did not meet the definition of human subjects

research per the determination by Dallas County Health and Human Services, Maryland Department of Health and City of Milwaukee Health Department. Each of the 4 labs tested between 40-52 randomly selected residual swab specimens collected for HSV testing. Samples were de-identified and stored at −80°C for 2 weeks to 3 months prior to testing.

GUD M-PCR testing at LRRB and PHLs

GUD M-PCR was performed as previously described with some modifications.[7] Briefly, DNA was extracted manually at LRRB using the QIAamp DNA Blood Mini Kit (Qiagen). PHL1 & PHL4 used the MagNA Pure Compact System (Roche Life Science) for DNA extraction and PHL 2 & 3 used the NucliSens® easyMAG® System (bioMerieux). LRRB provided PCR reagents and controls to the PHLs. The primer and probe sequences used for *H. ducreyi*, *T. pallidum*, HSV 1&2, and Human ribonuclease P (positive control) were described previously.[7] Real-time PCR was performed on a QuantStudio™ Dx System (ThermoFisher Scientific) at PHL1 and PHL3 while the other two PHLs used a ABI 7500 Fast Dx (Applied Biosystems Inc), and a Rotor-Gene Q (Qiagen) was used at LRRB. The PCR cycling conditions included an initial hold cycle at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 20 s and primer annealing and extension at 60 °C for 1 min. Positive control DNA (*H. ducreyi*, *T. pallidum*, and HSV) and no template controls (NTC) were used in each run for quality control.

Data analysis

The results were analyzed with SPSS (IBM®SPSS for Windows, Version 21.0). Overall percent agreement was calculated as (true positive plus true negative)/total number. True positive/negative refers to the result that agrees with LRRB test result. The Cohen's kappa coefficient (κ) was used to measure the agreement between testing at LRRB and PHLs.

Results

Characteristics of clinical specimens

The majority of specimens from PHL1, PHL2 and PHL4 were from males, 61.5%, 55% and 70%, respectively, while the specimens tested at PHL3 were predominantly from females (79.5%). The lesion swabs were collected from a variety of sites including penile (32%), vaginal (19%), urethral lesions (13%), oral (4.3), labial (3.2%), rectal (3.2%), non-genital skin (2.7%). Other sites include scrotal, pubic, buttock, perineum, and cervical. There were 35 (19%) specimens with unknown site of lesion.

Detection of pathogens by GUD M-PCR

As shown in Table 1, PHL1 identified 31/52 (59.6%) specimens as HSV positive with two discordant results. The overall agreement between PHL1 and LRRB for HSV was 96.2%. PHL2 detected 20/40 (50%) specimens as HSV positive, which had 100% agreement with LRRB results. PHL3 identified 43/44 (97.7%) HSV positive specimens. There were 2 discordant results which gave 95.5% agreement between the PHL3 and LRRB. PHL4 identified 19/50 (38%) as HSV positive with three discordant results, and the overall agreement between PHL4 and LRRB was 94%. The overall kappa value between the PHLs

and LRRB was 0.922, which suggests high agreement for the HSV component of the GUD M-PCR between LRRB and the four PHLs.

T. pallidum was detected in 7/51 (13.7%) specimens in PHL1, while testing at LRRB showed 10 positive specimens (Table 1). The overall agreement between PHL1 and LRRB for syphilis was 94.1%. *T. pallidum* was detected in 2/40 (5%) specimens both at PHL2 and LRRB, reflecting 100% agreement. *T. pallidum* was not detected in any specimen from PHL3 and PHL4 onsite or at LRRB. The overall kappa value for *T. pallidum* testing was 0.849, which suggests good agreement between PHLs and LRRB. In addition, PHL1 and PHL2 each had one specimen testing positive for both *T. pallidum* and HSV, which was confirmed by LRRB. None of the specimens tested positive for *H. ducreyi*.

Discussion

In this study, we evaluated the field performance of a GUD M-PCR assay at four PHLs. Overall, the assay performed well, with a 94-100% overall percent agreement and 0.922 Cohen's kappa value (high agreement) between LRRB and 4 PHLs for HSV testing. *T. pallidum* testing demonstrated overall percentage agreement of 94.1-100% and 0.849 kappa value (good agreement) between LRRB and PHLs. Our results show that the M-PCR assay can be successfully implemented in state and local PHLs and is potentially useful for the diagnosis of multiple sexually transmitted GUD infections.

The M-PCR assay identified *T. pallidum* in 12 (6.5%) specimens initially submitted for HSV testing. Although these results are encouraging, it's unclear if any of these patients were diagnosed with syphilis. Interestingly, two of the specimens had concomitant infections for HSV and *T. pallidum* identified by M-PCR. *H. ducreyi* was not detected in any of the study specimens; however, it is unlikely any of the swabs were collected from suspected chancroid cases since only a few cases of chancroid were reported in 2019 in the US. These cases were most likely based on clinical diagnosis as laboratory testing for *H. ducreyi* is rarely done.[2]

Commercial NAATs for *T. pallidum* or multiple sexually transmitted pathogens in GUD samples in the US are not widely available and none are FDA-cleared.[6] Quest Diagnostics offers a CLIA compliant *T. pallidum* PCR testing for genital lesion specimens. [8] Several multiplex NAATs are available for clinical use in Europe which are similar to the GUD M-PCR detecting *T. pallidum* and HSV. [9,10] While the results of these tests seem to be encouraging, further validation in a clinical setting with a larger sample size is imperative.

Our study has some limitations. We used a convenience sample of residual specimens collected from patients suspected of HSV infection and there may have been missed opportunities for diagnosis of *T. pallidum* infection. Sample size in each lab was limited by available funds. We were also not able to compare performance of the M-PCR assay with other FDA-cleared NAATs for detection of HSV and our assay does not differentiate between HSV-1&2. A few discordant results for HSV and *T. pallidum* were seen between the PHLs and LRRB possibly due to different testing platforms, but they could not be resolved due to the study design and/or insufficient specimen for repeat testing. None of the discordant specimens were co-infected. In light of the fact that chancroid is restricted

to rare sporadic cases in the US, we are currently modifying our assay for detection and differentiation of HSV 1&2, and *T. pallidum*, and are looking into the possibility of implementing the assay in PHLs. In summary, a NAAT for the detection of *T. pallidum* alone or both HSV and *T. pallidum* is urgently needed in the US for patients presenting with anogenital lesions. Our evaluation has demonstrated that the GUD M-PCR assay can be successfully implemented in PHLs and offer improved diagnostic options particularly for syphilis, which will lead to timely patient management.

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Data availability statement:

All data relevant to the study are included in the article or uploaded as supplementary information.

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Key messages

- Twelve suspected syphilis cases were identified in specimens collected for HSV testing.
- CDC developed TaqMan-based GUD M-PCR assay performed well in PHLs and could be implemented in state and local public health laboratories.
- The GUD M-PCR assay could be a valuable tool for timely diagnosis of multiple sexually transmitted GUD infections.

Table 1.

HSV and *T. pallidum* test results (Created by the authors).

Study site	HSV-PHL			HSV-CDC		Total	Overall percent agreement	Cohen's κ coefficient
	Positive	Negative	Discordant Result	Positive	Negative			
PHL1	31	21	2	31	21	52	96.1%	0.922
PHL2	20	20	0	20	20	40	100%	
PHL3	43	1	2	41	3	44	95.5%	
PHL4	19	31	3	18	32	50	94%	
Study site	***T. pallidum*-PHL**			***T. pallidum*-CDC**		**Total**	**Overall percent agreement**	**Cohen's κ coefficient**
	Positive	**Negative**	**Discordant Result**	**Positive**	**Negative**			
PHL1*	7	44	3	10	41	51	94.1%	0.849
PHL2	2	38	0	2	38	40	100%	
PHL3	0	44	0	0	44	44	100%	
PHL4	0	50	0	0	50	50	100%	

* One specimen from PHL1 was indeterminate for *T. pallidum* and excluded from the analysis.