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Original article

Evaluation of four commercial real-time PCR assays for the detection of lymphogranuloma venereum in *Chlamydia trachomatis*-positive anorectal samples

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ABSTRACT

Objectives: Lymphogranuloma venereum (LGV) is a sexually transmitted infection (STI) caused by *Chlamydia trachomatis* (CT) genovars L. The identification of LGV is of therapeutic interest because treatment requires 3 weeks of doxycycline compared with 1 week for infection with a non-L strain. The aim of this study was to evaluate the performance of four commercial real-time PCR kits in comparison with the reference methods used for LGV diagnosis by the French National Reference Centre (NRC) for bacterial STIs.

Methods: A total of 215 French CT-positive anorectal specimens collected consecutively in 2017 were used (66 LGV and 149 non-LGV). Among these, 92 were collected from symptomatic men who have sex with men (MSM) and 123 from asymptomatic MSM using pre-exposure prophylaxis. Four commercial assays were evaluated; a single-plex assay RealCycler CHSL kit (Progenie Molecular), tested on all the specimens, and three multiplex kits, the RealCycler Universal ULCGEN (Progenie Molecular), the Allplex Genital Ulcer Assay (Seegene) and the VIASURE *Haemophilus ducreyi* + CT LGV Real Time PCR Detection kit (CerTest Biotec), tested on the 92 samples from symptomatic MSM. Clinical performance was determined in comparison to the in-house real time PCR targeting the *pmp*H and the *omp*A gene sequencing.

Results: Overall agreement ranged between 91.3% and 100% (95% CI 83.7–100%) with very good Kappa index values (>0.8). The clinical sensitivities and specificities varied between 91% and 100% (95% CI 80.8–100%), and 97% and 100% (95% CI 87.1–100%), respectively, with some kits performing better than others.

Discussion: The four assays showed very good performance for the detection of LGV on anorectal specimens. **Arabella Touati, Clin Microbiol Infect 2021;27:909.e1–909.e5**

Introduction

Lymphogranuloma venereum (LGV) is a sexually transmitted infection (STI) caused by *Chlamydia trachomatis* (CT) genovars L1 to L3. Since 2003, the anorectal form of LGV has been endemic among European men who have sex with men (MSM), particularly in those who are HIV positive [1–3]. There is no evidence of LGV transmission within the heterosexual population, and this infection appears to be restricted to the MSM population [4–6]. CT genovars L2b and L2 are the causative strains in most European cases [1,7]. The identification of L genovars of CT is of epidemiological and therapeutic interest and should be performed early (preferably within 1 week) because LGV treatment requires 3 weeks of doxycycline compared with 1 week for an infection with a non-L genovar. It is also required for preventing long-term consequences of infection, as well as restricting secondary spread to sex partners [8].

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Since LGV testing kits were not commercially available until recently, the detection of LGV strains was performed using 'in-house' nucleic acid amplification tests (NAATs). The first genotyping NAATs consisted of PCR or nested PCR with endonuclease restriction [9]. In 2005, a real-time PCR assay was developed targeting the unique 36-bp deletion of the *pmp*H gene, which is specific to all LGV genovars [10]; this assay was later adapted and improved [11–14]. Most real-time PCR assays used for LGV detection are based on TaqMan or High Resolution Melting technology, and target the *omp*A and/or the *pmp*H genes [11–13,15,16].

According to European guidelines on LGV management, ideally, routine LGV molecular diagnostics should be available in all European countries, where they could be centralized at some national reference or similar type of expert laboratory [1]. However, in practice, LGV diagnosis is not available in all settings. In France, LGV detection is performed only by the National Reference Centre (NRC) for bacterial STIs. Laboratories throughout French metropolitan areas perform routine testing for the CT detection in anorectal specimens and then send positive specimens to the NRC for molecular diagnosis of LGV [3]. At a national scale, the NRC is attempting to satisfy the growing demand for LGV diagnosis in CT-positive anorectal samples. Recently, four commercial real-time PCR kits (one single-plex and three multiplex) were developed. The release of these kits will allow more laboratories to implement routine LGV diagnosis. However, there is a lack of data about the performances of theses kits. Besides, the multiplex commercial kits are intended for the diagnosis of genital ulcerative disease by detecting up to 6 STI pathogens including L genovars of CT.

Because anorectal infection is the most commonly reported clinical manifestation of LGV among MSM, we evaluated the performance of these four assays on anorectal CT-positive samples collected by the French NRC for bacterial STIs.

Methods

Study population and specimens

To evaluate the kits, we used a total of 215 CT-positive anorectal specimens from 92 French MSM with anorectal symptoms (49 LGV and 43 non-LGV) and 123 asymptomatic French MSM using pre-exposure prophylaxis (PrEP) (17 LGV and 106 non-LGV). The specimens were conserved at −80°C and collected consecutively in 2017.

Ethics approval

The French national sentinel surveillance for anorectal *C. trachomatis* infections was approved by the French Data Protection Authority (CNIL, no. 10.362) and data were collected after written informed consent was obtained from the patients.

Methods used at the NRC for LGV diagnosis

DNA extraction from specimens was performed using the MagNA Pure 96 DNA and Viral NA Small Volume kit and the MagNA Pure 96 instrument (Roche Diagnostics, USA). All anorectal specimens contained an extraction and inhibition real-time PCR internal control (DICD-CY-L100; Diagenode Diagnostics, Belgium) used for the validation of amplification of the in-house real-time PCR.

The NRC for bacterial STIs uses a genovar L-specific real-time single-plex PCR assay targeting the *pmp*H gene [10], in which all LGV genovars possess a unique gap of 36 bp. A positive result confirms the diagnosis of LGV, whereas a negative result excludes it. The gold standard for genovar confirmation is complete nucleotide sequence analysis of the *omp*A gene [17,18]. Thus, in addition to the results of the real-time PCR targeting the *pmp*H gene, sequencing of the *omp*A gene [9] was performed on all clinical specimens tested in this study. The results of both techniques were concordant for all specimens; therefore, both methods were used as reference methods.

To solve potential discrepancies between the assays, we sequenced a 488-bp fragment of *pmp*H gene, encompassing the 36-bp deletion specific to L-genovars. We designed the following primers: pmpH350F (5′-CTTGCGGAGAAAAGGGAATGA-3′) and pmpH837R (5′-GTATGCTGTGTTCCCTCGGA-3′).

Evaluated real-time PCR kits

We evaluated one single-plex and three multiplex commercial assays (Tables 1 and 2). The single-plex kit was the RealCycler® Universal CHSL-U/CHSL-G kit (Progenie Molecular, Spain), which detects only L genovars of CT. This assay was evaluated on the 215 CT-positive anorectal specimens from 92 symptomatic MSM and 123 asymptomatic MSM PrEP users. The three multiplex kits were (a) the RealCycler® Universal ULCGEN-U/ULCGEN-G (Progenie Molecular, Spain), which detects the Herpes Simplex Virus (HSV), *Treponema pallidum* and L genovars of CT, (b) the VIASURE *H. ducreyi* + CT LGV Real-Time PCR Detection Kit (CerTest BIOTEC, Spain), which detects *H. ducreyi* and L genovars of CT, and (c) the Allplex™ Genital Ulcer Assay (Seegene, South Korea), which detects HSV-1, HSV-2, *H. ducreyi*, cytomegalovirus (CMV), *T. pallidum* and L genovars of CT. These assays were tested on the 92 anorectal specimens from symptomatic MSM.

All tests were performed according to the manufacturer's instructions. Amplification was carried out on a CFX96™ real-time PCR system (Bio-Rad® Laboratories, France). For LGV diagnosis, the CHSL, the ULCGEN and the Viazure assays use Taqman® probes labelled with fluorophores that emit a fluorescence in the case of amplification. The Allplex assay exhibits Seegene's proprietary MuDT™ technology, which provides multi-Ct values in a single fluorescence channel without melt curve analysis on a real-time PCR instrument. Data were interpreted using CFX Manager™ 1.6 software (Bio-Rad) according to the manufacturer's instructions, with the exception of the Allplex assay, for which data were exported from the CFX Manager™ software and analysed using the Seegene Viewer software provided by the manufacturer.

All assays have been validated on urogenital samples, except for the Viazure assay which was also validated on anorectal specimens.

Statistical analyses

The performance of each assay was determined by calculating its clinical sensitivity and specificity, as well as overall per cent agreement values, with 95% confidence intervals (CIs), using the results of the in-house real-time PCR assay targeting the *pmp*H gene and *omp*A gene sequencing as reference methods. Concordance between the results of the commercial kits and those of the reference methods was analysed using Cohen's kappa (κ) index. Statistical analyses were performed using the BiostaTGV website (https://marne.u707.jussieu.fr/biostatgv/).

Results

We tested a total of 215 CT-positive anorectal specimens (66 LGV and 149 non-LGV) with the CHSL single-plex kit for LGV detection. An invalid result (internal control not amplified) was obtained for two samples, which were excluded from the subsequent calculation of diagnostic performance values (Table S1). Of the 66 LGV-positive specimens, the CHSL single-plex assay detected L genovars of CT in 65 specimens, showing an overall agreement of 98.6%

Table 1
Clinical performance of the four commercial kits for the detection of *C. trachomatis* genovars L

Commercial kit	Specimens tested n = 215(reference methods)			Sensitivity % (95% CI)	Specificity % (95% CI)	Overall agreement % (95% CI)	Kappa index
	LGV (n = 66)	Non LGV (n = 149)	Invalid IC				
RealCycler® Universal CHSL-U/CHSL-G Pathogen detected: CT genovars L	65	147	2	98.4 (91.9–99.7)	100 (97.4–100)	98.6 (95.9–99.5)	0.988
	Specimen tested n = 92 (refrence methods)						
	LGV (n = 49)	Non LGV (n = 43)	Invalid IC				
RealCycler® Universal ULCGEN-U/ULCGEN-G Pathogens detected: HSV, CT genovars L, *T. pallidum*	47	42	1	95.92 (86.2–98.8)	100 (91–100)	96.7 (90.8–98.8)	0.978
VIASURE *H. ducreyi* + *C. trachomatis* (LGV) Real Time PCR Detection kit Pathogens detected: *H. ducreyi* + CT genovars L	49	43	0	100 (92.7–100)	100 (91.8–100)	100 (95.9–100)	1
Allplex™ Genital Ulcer Assay Pathogens detected: HSV1, HSV2, *H. ducreyi,* CMV, CT genovars L, *T. pallidum,* VZV	45	39	3	91 (80.8–96.7)	97 (87.1–99.5)	91.3 (83.7–95.5)	0.887

CI, confidence interval; CT, *Chlamydia trachomatis*; IC, internal control; LGV, lymphogranuloma venereum; HSV, Herpes Simplex Virus; VZV, Varicella Zoster Virus; CMV, Cytomegalovirus.

% (95% CI 95.9–99.5%) with the reference methods (Table 1) and a clinical sensitivity and specificity of 98.4% (95% CI 91.9–99.7%) and 100% (95% CI 97.4–100%), respectively. The single-plex kit showed equivalent diagnostic performance for specimens from symptomatic and asymptomatic MSM, with 48/49 and 17/17 detected as LGV positive, respectively. Overall agreement with the reference methods was 98.9% (95% CI 94.1–99.8%) and 98.3% (95% CI 94.2–99.5%), respectively, and Kappa index was >0.8. Clinical sensitivity ranged between 97.9% and 100% (CI 95% 81.4–100%) respectively; the specificity was 100% (95% CI 91.8–100%) for both populations.

The three multiplex kits were tested on the 92 CT-positive anorectal specimens from symptomatic MSM (49 LGV and 43 non-LGV). Overall, three samples were invalid by the Allplex assay and one by the ULCGEN kit; these specimens were excluded from the subsequent analysis (Table S1). Among the 49 LGV-positive specimens, 47, 49 and 45 were detected by the ULCGEN, Viazure and Allplex kits, respectively (Table 1). The clinical sensitivity was 95.9%, 100% and 91% (95% CI 80.8–100%) for the ULCGEN, the Viazure and the Allplex kits, respectively. The clinical specificity of the ULCGEN and Viazure kits was 100% (95% CI 91–100%), whereas that of the Allplex assay was 97% (95% CI 87.1–99.5%). The overall agreement of the three multiplex commercial kits ranged between 91.3% and 100% (95% CI 83.7–100%). However, there was no significant difference in the performance of the different assays, as the 95% CIs of sensitivity and specificity overlapped, and Kappa index was very good (>0.8) for all three assays.

Discrepant results were found in eight cases involving six anorectal specimens (Table 3). In seven cases, corresponding to five specimens, a LGV-positive result was expected according to the reference methods but was found negative by at least one assay: one specimen was negative with the ULCGEN kit, three specimens were negative with the Allplex kit and one specimen was negative with the CHSL, the ULCGEN and the Allplex assays. The eighth case was found to be LGV negative with the reference methods (negative with the in-house real time PCR, genovar E according to *omp*A gene sequencing) but was positive by the Allplex kit (cycle threshold value of 36.27). These discrepancies were analysed by sequencing the *pmp*H gene fragment. Results showed that the commercial assays yielded false-negatives in the first seven cases, since the sequencing confirmed the presence of the 36-bp gap. The eighth case was confirmed as a false positive, with no deletion specific to LGV strain found in this specimen.

Discussion

The clinical performance of the kits evaluated in this study was determined compared with the reference methods used at the NRC (i.e. the in-house real time PCR targeting the *pmp*H gene and the *omp*A gene sequencing). The four assays showed very good agreement ($\kappa > 0.8$) with the reference methods, and equivalent performance with overlapping 95% CIs, despite a lower but non-significant sensitivity and specificity for the Allplex assay (Table 1). Notably, the single-plex kit was as effective for samples

Table 2
Handling, testing duration, and costs of the evaluated commercial kits

Commercial kit	No. of pathogens detected	LGV target	No. of reagents per reaction mixture	Reagents	Stability (°C)	IC	Run time (min)	Positive and negative controls	Data Analysis	CE marked	Price per reaction Excl. taxes (€) Catalogue Price
RealCycler® Universal CHSL-U/CHSL-G	1	*pmp*H	1	Frozen	18 months (−18 ~ −25)	Included	104	PC	CFX Manager (version 1.6)	yes	16.99
RealCycler® Universal ULCGEN-U/ULCGEN-G	3	*pmp*H	1	Frozen	18 months (−18 ~ −25)	Included	104	PC	CFX Manager (version 1.6)	yes	20.7
VIASURE *H. ducreyi* + CT (LGV) Real Time PCR Detection kit	2	*pmp*H	1	8-well strips lyophilized	24 months (2 ~ 40)	Included	90	PC+NC	CFX Manager (version 1.6)	In progress	13.02
Allplex™ Genital Ulcer Assay	7	NS	3	Frozen	12 months (-20)	Included	150	PC	Seegene Viewer	yes	42.62

LGV, lymphogranuloma venereum; NS, not specified; IC, internal control; PC, positive control; NC, negative control.

Table 3
Summary of discrepant results between the commercial assays and reference methods

Specimen	Reference methods (cycle threshold value)	Commercial kits (cycle threshold value)				*pmp*H sequencing (position 350 to 837)
		RealCycler® Universal CHSL	RealCycler® Universal ULCGEN	VIASURE *H. ducreyi* + *C. trachomatis* (LGV) Real Time PCR Detection kit	Allplex™ Genital Ulcer Assay	
1259	Positive (36.77)	Positive (35.27)	Negative	Positive (37.65)	Positive (33.73)	Presence of 36 bp-gap
29	Positive (36.88)	Positive (36.04)	Positive (36.26)	Positive (34.55)	Negative	Presence of 36 bp-gap
461	Positive (36.3)	Positive (34.3)	Positive (34.46)	Positive (34.59)	Negative	Presence of 36 bp-gap
376	Positive (36)	Positive (34.57)	Positive (34	Positive (36.02)	Negative	Presence of 36 bp-gap
1202	Positive (37)	Negative	Negative	Positive (38.59)	Negative	Presence of 36 bp-gap
292	Negative	Negative	Negative	Negative	Positive (36.27)	No gap

from symptomatic patients as for samples from asymptomatic patients.

In a recent study, compared with PCR restriction fragment length polymorphism results, the Allplex Genital ulcer assay and the VIASURE assay showed an overall agreement of 97.26% and 97.9%, respectively, for LGV detection on anorectal specimens [19]. Compared with each other, both commercial assays had equivalent performance with a kappa index of 0.98 [19]. However, that study used a non-standard reference method for LGV diagnosis. In the present study, the overall agreement of these two commercial assays was of 91.3% and 100%, respectively.

Six samples, all non-LGV, were invalid (internal control not amplified): three by the Allplex assay, one by the ULCGEN assay and two by the CHSL assay. In each case, the result was only invalid for one assay. Therefore, we did not repeat the analysis for these six specimens because they were validated by both reference methods.

Discrepant results were verified by sequencing a fragment of the *pmp*H gene; we confirmed that in seven cases, three commercial assays yielded false-negative results (CHSL kit $n = 1$, ULCGEN kit $n = 2$, Allplex kit $n = 4$), and in one case the Allplex assay reported a false-positive result (Table 3). One possible explanation for these seven false negatives is that the bacterial load may have been very low in these samples (Ct ranged between 36 and 38 with the in-house real-time PCR) and was therefore not detected by either test.

The four commercial assays were not developed for application in the same clinical settings. Multiplex kits are intended for the diagnosis of genital ulcerative disease and therefore were designed for symptomatic patients. For this reason, we chose to test the multiplex kits only on patients presenting symptoms. In contrast, the single-plex CHSL kit is intended for use as a second-line diagnostic method, to detect LGV in clinical specimens that have already been found to be CT positive. This kit can be used independently of the symptomatology, knowing that about 25% of the anorectal LGV infections are asymptomatic in MSM [1]. However, according to the European guideline on LGV management, a window of at least 2 weeks after exposure should be considered in this case [1].

We also assessed additional criteria such as the technology, handling, workflow, cost and stability of the assays. All of the evaluated kits have a short workflow sequence, with a ready-to-use reagent, except for the Allplex kit, which requires the mixture of three reagents (Table 2). In the Viazure assay, the PCR reaction mix is stabilized in a lyophilized format inside eight-well strips, allowing storage at room temperature and a very broad expiration date (up to 24 months), whereas the reagents of the remaining three assays are frozen and the expiration date is shorter (12–18 months).

The kits have in common to target the *pmp*H gene for the detection of L genovar, except the Allplex assay for which it is not specified. Moreover, the four assays included positive and internal controls to validate amplification. The Allplex assay also provides an internal control to be used with urine specimens, allowing confirmation of the nucleic acid extraction procedure and identification of PCR inhibition. Depending on the kit, the results are available within 90–150 min (Table 2).

Regarding marketing and distribution, all of the assays are certified for *in vitro* diagnosis within the European market, except for the Viazure kit. The Viazure kit is the least expensive per reaction (catalogue price: €13.02) whereas an Allplex assay reaction is much more costly (€42.62), but detects six other pathogens responsible for genital ulcers, in addition to LGV.

In conclusion, the four commercial kits showed very good performance for the detection of LGV in anorectal specimens, validated by the use of an internal amplification control. Among the assays certified for European distribution, the RealCycler® Universal CHSL- U/CHSL-G kit showed the best overall agreement, clinical sensitivity and specificity, compared with the reference methods. As recommended, LGV routine molecular diagnostics should be available in all European countries, especially with the growing demand for HIV-positive MSM, or MSM who are eligible for PrEP [1]. The implementation of an easy-to-use commercial kit will make identification of LGV strains available to many laboratories. Despite showing the best performance among the assays evaluated in this study, the Viazure kit presently lacks European certification, and is therefore not yet eligible for routine implementation in European laboratories.

Transparency declaration

The authors declare that they have no conflicts of interest. No external funding was received.

Author contributions

B.d.B. designed the study. C.L.N. collected the epidemiological data and made the statistical analysis. A.T. carried out the experiments, analysed and interpreted the data, and drafted the article. O.P. and B.d.B. revised critically the article. C.B. revised and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.07.040.

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