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1 **Original Article**

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

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20 **Keywords**: LGV, *C. trachomatis*, real-time PCR, commercial kits, anorectal specimens.

21

22 **Abstract**

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

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

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

43 Conclusion: The four assays showed very good performance for the detection of LGV on
44 anorectal specimens.

45 **Introduction:**

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

57 Since LGV testing kits were not commercially available until recently, the detection of LGV
58 strains was performed using “in-house” nucleic acid amplification tests (NAATs). The first
59 genotyping NAATs consisted of PCR or nested PCR with endonuclease restriction [9]. In
60 2005, a real-time PCR assay was developed targeting the unique 36-bp deletion of the *pmpH*
61 gene, which is specific to all LGV genovars [10]; this assay was later adapted and improved
62 [11-14]. Most real-time PCR assays used for LGV detection are based on TaqMan or High
63 Resolution Melting technology, and target the *ompA* and/or the *pmpH* genes [11-13, 15, 16].
64 According to European guidelines on LGV management, ideally, routine LGV molecular
65 diagnostics should be available in all European countries, where they could be centralized at
66 some national reference or similar type of expert laboratory [1]. However, in practice, LGV
67 diagnosis is not available in all settings. In France, LGV detection is performed only by the
68 National Reference Centre (NRC) for bacterial STIs. Laboratories throughout French
69 metropolitan areas perform routine testing for the CT detection in anorectal specimens and

70 then send positive specimens to the NRC for molecular diagnosis of LGV [3]. At a national
71 scale, the NRC is attempting to satisfy the growing demand for LGV diagnosis in CT-positive
72 anorectal samples. Recently, four commercial real-time PCR kits (one single-plex and three
73 multiplex) were developed. The release of these kits will allow more laboratories to
74 implement routine LGV diagnosis. However, there is a lack of data about the performances of
75 these kits. Besides, the multiplex commercial kits are intended for the diagnosis of genital
76 ulcerative disease by detecting up to 6 STI pathogens including L genovars of CT.
77 Because anorectal infection is the most commonly reported clinical manifestation of LGV
78 among MSM, we evaluated the performance of these four assays on anorectal CT-positive
79 samples collected by the French NRC for bacterial STIs.

80 **Methods**

81 **Study population and specimens**

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

86 **Ethical approval**

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

90 **Methods used at the NRC for LGV diagnosis**

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

95 of the in-house real-time PCR.

96 The NRC for bacterial STIs uses a genovar L-specific real-time single-plex PCR assay
97 targeting the *pmpH* gene [10], in which all LGV genovars possess a unique gap of 36 bp. A
98 positive result confirms the diagnosis of LGV, whereas a negative result excludes it. The gold
99 standard for genovar confirmation is complete nucleotide sequence analysis of the *ompA* gene
100 [17, 18]. Thus, in addition to the results of the real-time PCR targeting the *pmpH* gene,
101 sequencing of the *ompA* gene [9] was performed on all clinical specimens tested in this study.
102 The results of both techniques were concordant for all specimens; therefore, both methods
103 were used as reference methods.

104 To solve potential discrepancies between the assays, we sequenced a 488 bp-fragment of
105 *pmpH* gene, encompassing the 36 bp-deletion specific to L-genovars. We designed the
106 following primers : *pmpH350F* (5'-CTTGCGGAGAAAAGGGAATGA-3') and *pmpH837R*
107 (5'-GTATGCTGTGTTCCCTCGGA-3').

108 **Evaluated real-time PCR kits**

109 We evaluated one single-plex and three multiplex commercial assays (Tables 1 and 2):

110 - The single-plex kit was the RealCycler[®] Universal CHSL-U/CHSL-G kit (Progenie
111 Molecular, Spain), which detects only L genovars of CT. This assay was evaluated on the 215
112 CT-positive anorectal specimens from 92 symptomatic MSM and 123 asymptomatic MSM
113 PrEP users.

114 - The three multiplex kits were i/ the RealCycler[®] Universal ULCGEN-U/ULCGEN-G
115 (Progenie Molecular, Spain), which detects the Herpes Simplex Virus (HSV), *Treponema*
116 *pallidum* and L genovars of CT, ii/ the VIASURE *H. ducreyi* + CT LGV Real-Time PCR
117 Detection Kit (CerTest BIOTEC, Spain), which detects *Haemophilus ducreyi* and L genovars
118 of CT, and iii/ the Allplex[™] Genital Ulcer Assay (Seegene, South Korea), which detects

119 HSV-1, HSV-2, *H. ducreyi*, cytomegalovirus (CMV), *T. pallidum* and L genovars of CT.
120 These assays were tested on the 92 anorectal specimens from symptomatic MSM.
121 All tests were performed according to the manufacturer's instructions. Amplification was
122 carried out on a CFX96™ Real-time PCR system (Bio-Rad® Laboratories, France). For LGV
123 diagnosis, the CHSL, the ULCGEN and the Viazure assays use Taqman® probes labeled with
124 fluorophores that emit a fluorescence in the case of amplification. The Allplex assay exhibits
125 Seegene's proprietary MuDT™ technology, which provides multi-Ct values in a single
126 fluorescence channel without melt curve analysis on a real-time PCR instrument. Data were
127 interpreted using CFX Manager™ 1.6 software (Bio-Rad) according to the manufacturer's
128 instructions, with the exception of the Allplex assay, for which data were exported from the
129 CFX Manager™ software and analysed using the Seegene Viewer software provided by the
130 manufacturer.
131 All assays have been validated on urogenital samples, except for the Viazure assay which was
132 also validated on anorectal specimens.

133 **Statistical analyses**

134 The performance of each assay was determined by calculating its clinical sensitivity and
135 specificity, as well as overall percent agreement values, with 95% confidence intervals (CIs),
136 using the results of the in-house real-time PCR assay targeting the *pmpH* gene and *ompA* gene
137 sequencing as reference methods. Concordance between the results of the commercial kits and
138 those of the reference methods was analysed using Cohen's Kappa (κ) index. Statistical
139 analyses were performed using the BiostaTGV website
140 (<https://marne.u707.jussieu.fr/biostatgv/>).

141 **Results**

142 We tested a total of 215 CT-positive anorectal specimens (66 LGV and 149 non-LGV) with
143 the CHSL single-plex kit for LGV detection. An invalid result (internal control not amplified)

144 was obtained for two samples which were excluded from the subsequent calculation of
145 diagnostic performance values (Table S1). Of the 66 LGV-positive specimens, the CHSL
146 single-plex assay detected L genovars of CT in 65 specimens, showing an overall agreement
147 of 98.6% (95% CI 95.9%-99.5%) with the reference methods (Table 1) and a clinical
148 sensitivity and specificity of 98.4% (95% CI 91.9%-99.7%) and 100% (95% CI 97.4%-
149 100%), respectively. The single-plex kit showed equivalent diagnostic performance for
150 specimens from symptomatic and asymptomatic MSM, with 48/49 and 17/17 detected as
151 LGV-positive, respectively. Overall agreement with the reference methods was 98.9% (95%
152 CI 94.1%-99.8%) and 98.3% (95% CI 94.2%-99.5%), respectively, and Kappa index was >
153 0.8. Clinical sensitivity ranged between 97.9% and 100% (95% CI 81.4%-100%) respectively;
154 the specificity was 100% (95% CI 91.8%-100%) for both populations.

155 The three multiplex kits were tested on the 92 CT-positive anorectal specimens from
156 symptomatic MSM (49 LGV and 43 non-LGV). Overall, three samples were invalid by the
157 Allplex assay and one by the ULCGEN kit; these specimens were excluded from the
158 subsequent analysis (Table S1). Amongst the 49 LGV-positive specimens, 47, 49, and 45
159 were detected by the ULCGEN, Viazure, and Allplex kits, respectively (Table 1). The clinical
160 sensitivity was 95.9%, 100% and 91% (95% CI 80.8%-100%) for the ULCGEN, the Viazure,
161 and the Allplex kits, respectively. The clinical specificity of the ULCGEN and Viazure kits
162 was 100% (95% CI 91%-100%), whereas that of the Allplex assay was 97% (95% CI 87.1%-
163 99.5%). The overall agreement of the three multiplex commercial kits ranged between 91.3%
164 and 100% (95% CI 83.7%-100%). However, there was no significant difference in the
165 performance of the different assays, as the 95% CIs of sensitivity and specificity overlapped,
166 and Kappa index was very good (> 0.8) for all three assays.

167 Discrepant results were found in eight cases involving six anorectal specimens (Table 3). In
168 seven cases, corresponding to five specimens, a LGV-positive result was expected according

169 to the reference methods but was found negative by at least one assay: one specimen was
170 negative with the ULCGEN kit, three specimens were negative with the Allplex kit, and one
171 specimen was negative with the CHSL, the ULCGEN and the Allplex assays. The eighth case
172 was found to be LGV-negative with the reference methods (negative with the in-house real
173 time PCR, genovar E according to *ompA* gene sequencing) but was positive by the Allplex kit
174 (cycle threshold value of 36.27). These discrepancies were analysed by sequencing the *pmpH*
175 gene fragment. Results showed that the commercial assays yielded false-negatives in the first
176 seven cases, since the sequencing confirmed the presence of the 36-bp gap. The eighth case
177 was confirmed as a false-positive, with no deletion specific to LGV strain found in this
178 specimen.

179 **Discussion**

180 The clinical performance of the kits evaluated in this study was determined in comparison to
181 the reference methods used at the NRC (i.e. the in-house real time PCR targeting the *pmpH*
182 gene and the *ompA* gene sequencing). The four assays showed very good agreement ($\kappa > 0.8$)
183 with the reference methods, and equivalent performance with overlapping 95% CIs, despite a
184 lower but non-significant sensitivity and specificity for the Allplex assay (Table 1). Notably,
185 the single-plex kit was as effective for samples from symptomatic patients as for samples
186 from asymptomatic patients.

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

193 Six samples, all non-LGV, were invalid (internal control not amplified): three by the Allplex

194 assay, one by the ULCGEN assay and two by the CHSL assay. In each case, the result was
195 only invalid for one assay. Therefore, we did not repeat the analysis for these six specimens
196 because they were validated by both reference methods.

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

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

212 We also assessed additional criteria such as the technology, handling, workflow, cost and
213 stability of the assays. All of the evaluated kits have a short workflow sequence, with a ready-
214 to-use reagent, except for the Allplex kit, which requires the mixture of three reagents (Table
215 2). In the Viazure assay, the PCR reaction mix is stabilized in a lyophilized format inside 8-
216 well strips, allowing a storage at room temperature and a very broad expiration date (up to 24
217 months), whereas the reagents of the remaining three assays are frozen and the expiration date
218 is shorter (12 to 18 months).

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

225 Regarding marketing and distribution, all of the assays are certified for *in vitro* diagnosis
226 within the European market, except for the Viazure kit. The Viazure kit is the least expensive
227 per reaction (catalogue price: €13.02) whereas an Allplex assay reaction is much more costly
228 (€42.62), but detects six other pathogens responsible for genital ulcers, in addition to LGV.

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

240

241

242 **Transparency declaration**

243 The authors have no conflicts of interest to declare.

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252

253 **Contributors:**

254 BdB designed the study. CLN collected the epidemiological data and made the statistical
255 analysis. AT carried out the experiments, analysed and interpreted the data, and drafted the
256 article. OP and BdB revised critically the article. CB revised and approved the final
257 manuscript.

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325

326 **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
	Specimens tested n = 92 (Reference methods)			Sensitivity % [95% CI]	Specificity % [95% CI]	Overall agreement % [95% CI]	Kappa index
	LGV (n= 49)	Non LGV (n=43)	Invalid IC				
RealCycler® Universal ULCGEN-U/ULCGEN-G Pathogens detected: HSV, CT genovars L, <i>T. pallidum</i>	47	42	1	95.92 [86.2-98.8]	100 [91-100]	96.7 [90.8-98.8]	0.978
VIASURE <i>H. ducreyi</i> + <i>C. trachomatis</i> (LGV) Real Time PCR Detection kit Pathogens detected: <i>H. ducreyi</i> + 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, <i>H. ducreyi</i> , CMV, CT genovars L, <i>T. pallidum</i> , VZV	45	39	3	91 [80.8-96.7]	97 [87.1-99.5]	91.3 [83.7-95.5]	0.887

327 CI: confidence interval, CT: *Chlamydia trachomatis*, IC: internal control, LGV: lymphogranuloma venereum, HSV: Herpes Single-plex Virus, VZV: Varicella

328 Zoster Virus, CMV: Cytomegalovirus

329 **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	<i>pmpH</i>	1	Frozen	18 months (-18 ~ -25)	Included	104	PC	CFX™ Manager (version 1.6)	yes	16.99
RealCycler® Universal ULCGEN-U/ULCGEN-G	3	<i>pmpH</i>	1	Frozen	18 months (-18 ~ -25)	Included	104	PC	CFX™ Manager (version 1.6)	yes	20.7
VIASURE <i>H.ducreyi</i> + CT (LGV) Real Time PCR Detection kit	2	<i>pmpH</i>	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

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

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

Specimen	Reference methods (cycle threshold value)	Commercial kits (cycle threshold value)				<i>pmpH</i> sequencing (position 350 to 837)
		RealCycler® Universal CHSL	RealCycler® Universal ULCGEN	VIASURE <i>H. ducreyi</i> + <i>C. trachomatis</i> (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 (34.22)	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

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