Lymphogranuloma venereum variant L2b-specific polymerase chain reaction: insertion used to close an epidemiological gap

S. P. Verweij[1], A. Catsburg[2], S. Ouburg[1], A. Lombardi[3], R. Heijmans[1], F. Dutly[4], R. Frei[5], S. A. Morré[1] and D. Goldenberger[5]

1) *Laboratory of Immunogenetics, Department of Pathology,* 2) *Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, the Netherlands,* 3) *Department of Clinical Microbiology, University Hospital L. Sacco, Milano, Italy,* 4) *IMD, Institute for Medical and Molecular Diagnostics Ltd, Zurich and* 5) *Division of Clinical Microbiology, University Hospital Basel, Basel, Switzerland*

Abstract

The management of the ongoing *lymphogranuloma venereum* epidemic in industrialized Western countries caused by *Chlamydia trachomatis* variant L2b still needs improvements in diagnosis, therapy and prevention. We therefore developed the first rapid *C. trachomatis* variant L2b-specific polymerase chain reaction to circumvent laborious *ompA* gene sequencing.

Keywords: *Chlamydia trachomatis*, diagnostics, L2b, LGV, lymphogranuloma venereum, MSM, RT-PCR, OmpA, PmpH, sequencing

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Corresponding author: S. A. Morré, VU University Medical Center, Department of Pathology, Laboratory of Immunogenetics (LoI), De Boelelaan 1117, 1081 HV, Amsterdam, the Netherlands
E-mail: samorretravel@yahoo.co.uk

Lymphogranuloma venereum (LGV) is caused by *Chlamydia trachomatis* serovars L1–L3. LGV is more invasive than infections caused by the oculo-genital *C. trachomatis* serovars D–K. It classically manifests itself as an inguinal syndrome, with genital ulceration, inguinal lymphadenopathy (buboes), and subsequent suppuration. But it can also cause a severe anorectal syndrome, with proctocolitis and hyperplasia of intestinal and perirectal lymphatic tissue [1]. LGV is endemic in Africa, southeast Asia and the Caribbean. It is a sporadic disease in Europe and North America.

In 2003, an LGV outbreak was reported in the Netherlands and other Western European countries among men who have sex with men (MSM). The European Surveillance of Sexually Transmitted Infections (http://www.essti.org) and the Centers for Disease Control and Prevention (http://www.cdc.gov) instigated warning and response systems to increase the awareness and the management of the LGV outbreak, but the outbreak is still ongoing [1–8].

In 2005, we identified a unique single-nucleotide mutation in the *ompA* gene of the LGV L2 serovar circulating among MSM. This serovariant was designated L2b [2]. Subsequently we developed a specific LGV real-time polymerase chain reaction (PCR) exploiting a unique deletion (36 bp) in all LGV serovars within the polymorphic membrane protein H (*pmpH*) gene [3].

In our laboratories in Amsterdam and Basel we get frequent epidemiology-based requests to identify the aetiological LGV serovar as the L2b variant. To identify the L2b mutation in the variable segment 2 of the *ompA* gene, we have to amplify and sequence this fragment. To avoid the high costs of sequencing and the need for sophisticated equipment, we developed an L2b-specific primer/probe set using fast and reliable real-time PCR techniques for identification of the L2b variant.

The Study

We sequenced the *pmpH* gene (2952 bp) of two serovariant L2b-containing clinical specimens (accession numbers EF534758 and EF612788), which were 100% identical, and compared them with a reference sequence from serovar L2. To our surprise we identified several unique differences (Figs 1 and 2). First, L2b has a single-nucleotide polymorphism at the second base where the previously developed LGV-specific probe binds, just besides the 36-bp deletion (Fig. 1). Based on this finding we adjusted our previously published [3] probe: instead of a C base, we incorporated a degenerated base (C/T) to be able to detect all LGV serovars and variants L1, L2, L2a, L2b and L3 adequately. Additionally, we identified a 9-bp insertion unique for L2b resulting in a repeat sequence $(TCT\ AGT\ AGT)_2$ (Fig. 2). These two sequence heterogeneities were then confirmed in another ten L2b-positive samples by sequencing. We verified whether the insertion is unique

	475	485	495	505	515	525
L2b	AACTCCGC T T	GC--------	----------	----------	--------TC	CAACAGTTAG
L1	AACTCCGC C T	GC--------	----------	----------	--------TC	CAACAGTTAG
L2	AACTCCGC C T	GC--------	----------	----------	--------TC	CAACAGTTAG
L3	AACTCCGC C T	GC--------	----------	----------	--------TC	CAACAGTTAG
Probe	C C T	GC			TC	CAACAGT
A	AACTCCGC C T	GCTCTAGATC	CATCCCCTAC	CGCTTCAAGC	TCTTCATCTC	CCACAGTCAG
B	AACTCCGC C T	GCTCCAGATC	CATCCCCTAC	CGCTTCAAGC	TCTTCATCTC	CCACAGTCAG
Ba	AACTCCGC C T	GCTCCAGATC	CATCCCCTAC	CGCTTCAAGC	TCTTCATCTC	CCACAGTCAG
C	AACTCCGC C T	GCTCCAGATC	CATCCCCTAC	CGCTTCAAGC	TCTTCATCTC	CCACAGTCAG
D	AACTCCTC C A	GCACCAGCAC	CAGCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
Da	AACTCCGC C T	GCTCCAGATC	CATCCCCTAC	CGCTTCAAGC	TCTTCATCTC	CCACAGTCAG
E	AACTCCTC C A	GCACCAGCAC	CAGCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
SW-E	AACTCCTC C A	GCACCA----	--GCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
F	AACTCCTC C A	GCACCAGCAC	CAGCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
G	AACTCCTC C A	GCACCAGCAC	CAGCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
H	AACTCCTC C A	GCACCAGCAC	CAGCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
I	AACTCCTC C A	GCACCA----	--GCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
Ia	AACTCCTC C A	GCACCAGCAC	CAGCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
J	AACTCCTC C A	GCACCA----	--GCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
Ja	AACTCCTC C A	GCACCA----	--GCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG
K	AACTCCTC C A	GCACCA----	--GCTCCTGC	TGCTTCAAGC	TCTTTATCTC	CAACAGTTAG

FIG. 1. LGV-specific probe: alignment of partial pmpH gene of *C. trachomatis* serovars. L2b sequence used as reference (accession numbers EF534758 and EF612788). Nucleotide numbers are relative to the start codon. The mutation in L2b is marked by the red rectangle. The first four lines represent the LGV serovars. Probe represents the LGV-specific MGB-probe, A–K represent the ocular and urogenital *C. trachomatis* serovars. SW-E is the Swedish variant of serovar E. The dashed lines within the LGV serovars represent the LGV-specific deletion sequence.

	1845	1855	1865	1875	1885	1895	1905	1915
L2b	TGGGATCGCT	GACGATTCCT	TTTGTTACCC	TATCTTCTAG	TAGTTCTAGT	AGTGCTAGTA	ACGGGGTTAC	AATGAAGCGT
Forward	TCGCT	GACGATTCCT	TTTGTT					
Probe				CTTCTAG	TAGTTCTAGT	AGTGCT		
Reverse						TA	ACGGGGTTAC	AATGAAGCG
L1	TGGGATCGCT	GACGATTCCT	TTTGTTACCC	TATCTTCTAG	TAGT------	---ACTAGTA	ACGGGGTTAC	AATGAAGCGT
L2	TGGGATCGCT	GACGATTCCT	TTTGTTACCC	TATCTTCTAG	TAGT------	---ACTAGTA	ACGGGGTTAC	AATGAAGCGT
L3	TGGGATCGCT	GACGATTCCT	TTTGTTACCC	TATCTTCTAG	TAGT------	---ACTAGTA	ACGGGGTTAC	AATGAAGCGT
A	TGGGATCGCT	GACAATTCCT	TTTGTTACCC	TATCTTCTGT	CAAT---AAT	ACCAATAGTA	ATGGGGTTGT	AAAAACAGCT
B	TGGGATCGCT	GACAATTCCT	TTTGTTACCC	TATCTTCTGT	CAAT---AAT	ACCAATAGTA	ATGGGGTTGT	AAAAACAGCT
Ba	TGGGATCGCT	GACAATTCCT	TTTGTTACCC	TATCTTCTGT	CAAT---AAT	ACCAATAGTA	ATGGGGTTGT	AAAAACAGCT
C	TGGGATCGCT	GACAATTCCT	TTTGTTACCC	TATCTTCTGT	CAAT---AAT	ACCAATAGTA	ATGGGGTTGT	AAAAACAGCT
D	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
Da	TGGGATCGCT	GACAATTCCT	TTTGTTACCC	TATCTTCTGT	CAAT---AAT	ACCAATAGTA	ATGGGGTTGT	AAAAACAGCT
E	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
SW-E	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
F	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
G	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
H	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
I	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
Ia	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
J	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
Ja	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT
K	TGGGATCGCT	GACAATTCCT	TTTGTTACTC	TATCTTCTAG	TAGT------	---GCTAGTA	ACGGAGTTAC	AAAAAATTCT

FIG. 2. L2b-specific PCR: alignment of partial pmpH gene of *C. trachomatis* serovars. L2b sequence used as reference (accession numbers EF534758 and EF612788). Nucleotide numbers are relative to the start codon. The unique insertion of the L2b serovariant is located at relative position 1885–1893. The newly developed probe covers the repeat $(TCT\ AGT\ AGT)_2$. Forward, Reverse and Probe represent the primers and MGB-probe of the L2b-specific PCR, L1–L3 and A–K represent the *C. trachomatis* LGV and oculogenital serovars, respectively. The reverse primer depicted here is the reverse complimentary sequence of the actual reverse primer.

among all *C. trachomatis* serovars. Using BioEdit Sequence Alignment Editor we aligned the sequences of *pmpH* genes of 19 serovars/serovariants (NCBI accession number in brackets): A (AY184155), B (AY184156), Ba (AY184157), C (AY184158), D (AY184159), Da (AY967759), E (AY184160), Swedish variant E (SW-E; FN652779), F (AY184161), G (AY184162), H (AY184163), I (AY184164), Ia (AY967760), J (AY184165), K (AY184166), L1 (AY184167), L2 (AY184168) and L3 (AY184169). The analysis confirmed the unique nature of the insert of L2b within the *pmpH* gene (Fig. 2). Our sequence findings have also been confirmed by the recent publication of the entire L2b genome [9] (accession no. NC_010280). The following primers and probe were selected based on this unique insertion: L2b-F 5′ TCG CTG ACG ATT CCT TTT GTT 3′, L2b-R 5′ CGC TTC ATT GTA ACC CCG TTA 3′, and L2b MGB-probe 5′ VIC-CTT CTA GTA GTT CTA GTA GTG CT-MGB 3′. Standard TaqMan conditions with 45 cycles of 15 s at 95°C and 1 min at 60°C were used for PCR amplification.

We compared our old LGV probe with the adjusted LGV probe within the LGV-specific deletion region. We tested *C. trachomatis* L1, L2, L2a, L2b and L3 reference strains. Using titrated serial dilutions we found a slightly increased analytical sensitivity (factor 2–20) of our new LGV assay for the detection of L2b. Although we found this increased analytical sensitivity, every L2b case previously tested was detected by both the old and new LGV assay. No differences were observed for the other serovars tested, showing a well-defined LGV-specific PCR.

To determine specificity of the newly developed L2b-specific insertion probe, we firstly analysed different *Chlamydia* strains: *C. trachomatis* (serovars/serovariants, A, B, Ba, C, D, Da, D-, E, F, G, Ga, H, I, Ia, J, K, L1, L2, L2a, L3), *C. muridarum* (MoPn), *C. pneumoniae*, *C. pecorum* and *C. psittaci*. Secondly, we tested 31 microorganisms found in the perianal and urogenital regions and the oropharynx: *Bacteroides* spp. (*ao. fragilis*), *Clostridium* spp. (*ao. novyi*), *Faecalibacterium prausnitzii*, *Atopobium parvulum*, *Enterococcus faecalis*, *Propionibacterium acnes*, *Bordetella pertussis*, *Neisseria gonorrhoeae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Bartonella henselae*, *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*. Thirdly, we isolated DNA from *C. trachomatis*- and LGV-negative rectal swaps obtained from ten healthy men. Isolated DNA of these samples included (unknown) microorganisms commonly found in the rectum. Finally, we included a series of 60 *ompA*-based L2b-positive clinical isolates from Switzerland, the Netherlands and Italy (see Table 1 for an overview of the results).

The results showed that only the samples that had been previously diagnosed as L2b by *ompA* sequencing tested positive with the new insertion assay. There was no cross-reactivity with either the *Chlamydia* serovars/serovariants, including L2 and L2a, *Chlamydia* species or the other tested bacteria and clinical samples. In addition, we found equal sensitivity of the new L2b-specific and our adjusted LGV-specific PCR performing serial dilution tests. As expected, the general *C. trachomatis* plasmid PCR, targeting around ten cryptic plasmids, had a 10–50 times higher analytical sensitivity in serial dilutions.

TABLE 1. Overview of the LGV L2b test results

	L2b RT-PCR test results	
	Positive with L2b RT-PCR	**Negative with L2b RT-PCR**
C. trachomatis serovars A–K	–	All
C. trachomatis serovars L1, L2, L2a, L3	–	All
C. muridarum (MoPn), *C. pneumoniae*, *C. pecorum*, *C. psittaci*	–	All
31 commonly found microorganisms*	–	All
Ten rectal swaps of healthy men	–	All
60 L2b-positive samples	All	–

*These 31 microorganisms are commonly found in the urogenital and anorectal tract, or in the oropharynx.

Conclusions

The prevalence of LGV is still increasing in many European countries since the first reports of the LGV epidemic in 2003 [10]. Almost all strains causing this LGV epidemic are L2b [11–13]. Mapping the spread and prevalence of LGV serovariant L2b specifically may provide answers to important epidemiological questions that are needed for disease control and prevention. Therefore, a fast and highly accurate detection assay is a prerequisite. Our new LGV L2b test fulfills all these requirements and omits the laborious *ompA* sequencing step.

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Transparency Declaration

Conflicts of interest: nothing to declare.

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