Clinical and epidemiological characterization of a lymphogranuloma venereum outbreak in Madrid, Spain: co-circulation of two variants

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Abstract

The lymphogranuloma venereum (LGV) outbreak described in the Netherlands in 2003, increased the interest in the genotyping of *Chlamydia trachomatis*. Although international surveillance programmes were implemented, these studies slowly decreased in the following years. Now data have revealed a new accumulation of LGV cases in those European countries with extended surveillance programmes. Between March 2009 and November 2011, a study was carried out to detect LGV cases in Madrid. The study was based on screening of *C. trachomatis* using commercial kits, followed by real-time *pmpH*-PCR discriminating LGV strains, and finally *ompA* gene was sequenced for phylogenetic reconstruction. Ninety-four LGV infections were identified. The number of cases increased from 10 to 30 and then to 54 during 2009–2011. Incidence of LGV was strongly associated with men who have sex with men; but in 2011, LGV cases were described in women and heterosexual men. Sixty-nine patients were also human immunodeficiency virus (HIV) positive, with detectable viral loads at the moment of LGV diagnosis, suggesting a high-risk of co-transmission. In fact, in four patients the diagnosis of HIV was simultaneous with LGV infection. The conventional treatment with doxycycline was prescribed in 75 patients, although in three patients the treatment failed. The sequencing of the *ompA* gene permitted identification of two independent transmission nodes. One constituted by 25 sequences identical to the L2b variant, and a second node including 37 sequences identical to L2. This epidemiological situation characterized by the co-circulation of two LGV variants has not been previously described, reinforcing the need for screening and genotyping of LGV strains.

Keywords: co-circulation, L2, L2b, Lymphogranuloma venereum, Lymphogranuloma venereum-outbreak, Spain
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Introduction

Lymphogranuloma venereum (LGV) cases have rarely been diagnosed in industrialized countries, but in 2003 a small LGV outbreak was described in the Netherlands among men who have sex with men (MSM) [1]. Later, further outbreaks were rapidly communicated across many European countries [2]. As a consequence, alerts about the increase in LGV cases were communicated for the European Centre for Disease Prevention

and Control and the CDC [3,4] and surveillance programmes were implemented in several countries such as the Netherlands [5] and the UK (www.hpa.org.uk). During subsequent years, many more countries reported cases of LGV in Europe, Australia and the USA [6,7]. However, since 2009 the alarm has decreased and some authors predicted that LGV outbreaks would decline in the near future [8], even though new European regions continued to detect isolated cases of LGV in subsequent years [9]. There is limited epidemiological information about the evolution of the international outbreak, as only a few national surveillances are implemented and the published data refer only to high-risk populations. However, a new accumulation of cases is currently occurring in some countries with extended surveillance programmes. For instance, the UK Health Protection Agency (www.hpa.org.uk) and a Spanish region have detected an increase of ~ 115% in the last 2 years [10]. More worryingly, in the Netherlands this increase has reached 265% in the first 6 months of 2012 [11]. Moreover, the British Columbia Centre for Disease Control (www.bccdc.ca) and the National System of Epidemiological Surveillance of Mexico (www.dgepi.salud.gob.mx) have also described a sustained increase of LGV cases in recent years.

Since the description of the first LGV cases, researchers realized that the genotyping of LGV should be improved, because LGV spread could be linked to delay in diagnosis [12] and inappropriate duration of antibiotic treatment [13]. Moreover, strong association between LGV and human immunodeficiency virus (HIV) [3,4], and other sexually transmitted infections (STI) such as syphilis and hepatitis C virus have been documented [14]. Unfortunately, many laboratories still do not have genetic tools to discriminate LGV genovars from other genovars of *Chlamydia trachomatis*, suggesting that the cases of LGV could be underestimated [15]. In this epidemiological situation there are several reasons for increasing efforts for correct identification of LGV genovars.

Real-time PCR techniques were developed based on internal deletion of the *pmpH* gene [16], facilitating LGV surveillance [17]. The current recommendation for the diagnosis and surveillance of LGV suggests a screening stage based on commercial kits to detect all *C. trachomatis* infections, followed by real-time *pmpH*-PCR to discriminate between LGV and non-LGV strains and finally *ompA* gene sequencing as the most appropriate approach for phylogenetic reconstruction [18]. The aim of the present study was to detect the presence of LGV cases in Madrid, and if these genotypes were detected the sequencing of *ompA* gene could permit phylogenetic reconstructions to find a reason for the hypothetical increase in the number of LGV cases.

Methods

Collection of clinical strains

Two STI Units in Madrid, Spain, were involved in the present study. From March 2009 to November 2011 a total of 13 585 samples were collected from 8407 attendees, who came voluntarily for suspected STI as a consequence of sexual risk behaviour. The age range was 14–79 years; 4190 were women (49.8%), 59 (0.7%) were transsexuals and 4148 (49.5%) were men, of whom 3282 (78.9%) were MSM.

The criteria for collecting samples were based on clinical symptoms or sexual risk behaviours with or without symptoms. In women, the samples were obtained from cervix to rectum in the case of anal sex, in men, from rectum to urethra. The distribution of 13 585 samples was rectal (n = 3185), urethral (n = 2420), cervical (n = 5462) and pharyngeal (n = 2518). All samples were cultured or analysed for the presence of microorganisms related to sexually transmitted diseases, including *C. trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum* and HIV.

This study was approved by the Ethics Committee of Ramon y Cajal Hospital.

Laboratory diagnosis of *C. trachomatis* and LGV-serovars

Screening of *C. trachomatis* was based on two molecular commercial tests: Abbott Real Time CT/NG (Abbott Laboratories, Des Plaines, IL, USA) was performed in one STI Unit, and BD-ProbeTec™ ET CT/GC (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) in the second STI Unit, according to the manufacturer's recommendations. The samples yielding positive amplifications for *C. trachomatis* were anonymized and sent in a suitable transport medium from the diagnostic kit to the second laboratory for genotyping. In the second laboratory, a new DNA extraction was performed using Nuclisens easyMag (BioMérieux, Inc, Durham, NC, USA). Then, a specific real-time PCR to *C. trachomatis pmpH* gene where a 36 base-pair (bp) deletion occurs, was performed in all LGV serovars, as previously described [19,20]. In brief, a fragment of 60 bp was amplified using the primers LGV-F: 5′-CTGTGCCAACCTCATC ATCAA-3′ and LGV-R 5′-AGACCCTTTCCGAGCATCACT-3′ and as probe 5′-FAM-CCGCCTGCTCCAACAGTTAGTGATG-BHQ1 [21]. A complete description of the real-time PCR conditions can be found in the original publications [16,20]. Moreover, a specific detection of the *ompA* gene was performed in parallel with *pmpH* evaluation. An amplicon of 69 bp, between 124 and 196 positions, was detected. The combinations of primers and probe are also previously described [16]. Reference strain of *C. trachomatis* ATCC VR-902B was used as positive control for LGV in each real-time PCR assay. A presumptive diagnosis of

LGV infection was considered when the *pmpH*-PCR yielded positive amplification.

Sequence and phylogenetic analyses

To perform the phylogenetic analyses, 858 bp of *ompA* gene, encoding the major outer membrane protein, were sequenced in all LGV cases. The primers ompA-F: 5′-ATGAAA AAACTCT TGAAATCGG-3′ and ompA-R: 5′-ACTGTAACTGCGTATTT GTCTG-3′ were used following the previously published recommendations [22]. Sequences from all *C. trachomatis* LGV available at the National Center for Biotechnology database were downloaded (http://www.ncbi.nlm.nih.gov), including at least one representative of LGV variants described (Fig. 2). Sequences were edited with CHROMAS 2.33 software and aligned using CLUSTALX version 2.0 followed by manual editing. To discard sequencing and alignment errors all nucleotide sequences were translated to proteins to assure the absence of nonsense mutations. The sequences of *ompA* gene from detected LGV strains were used to reconstruct a median-joining network 4.6.1.0 (http://www.fluxus-technology.com).

Nucleotide sequence accession numbers

The *ompA* sequences described in this work have been deposited in GenBank with the accession numbers between: JX971886 and JX971960.

Statistical analysis

For exploring the associations between nominal-by-nominal measures (symptoms or not symptoms), chi-squared test or Fisher's exact test, when sample size was small, were used.

Results

Characterization of the recent LGV outbreak in Madrid

A total of 1239 of 13 585 specimens (9.1%) yielded a positive *C. trachomatis* result. Based on the *pmp*H gene amplification 94/1239 (7.6%) were further identified as LGV. Overall, LGV cases increased during the study period (120%, 2009–2010; 80% 2010–2011) (Fig. 1). They were more often detected in MSM (87/94, 92.5%), but in 2011, seven cases were diagnosed in the heterosexual population (four men and three women), suggesting that the outbreak is far from being controlled. As to country of origin, 56% were Spaniards, 37.3% were South Americans, 6.6% were non-Spanish Europeans and 1.3% were from other regions.

Identification of two transmission clusters responsible for the LGV outbreak

In 75/94 LGV strains an 858-bp fragment of the *ompA* gene was successfully sequenced. In 25/75 (33.3%) sequences were identical to *ompA* from L2b, 37/75 (49.3%) were identical to L2/L2f and the remaining 13/75 sequences (17.3%) were variants derived from them. The network phylogenetic tree revealed two major transmission nodes (Fig. 2). A node was constituted by 25 sequences identical to L2b, which has been implicated in the LGV worldwide outbreak [23]. The second node of transmission consisted of 37 sequences identical to the L2/L2f variant [21,24]. Moreover, ten new genotypes, carrying amino acid changes not previously described derived from L2/L2f and L2b, were also identified. According to the numbering of L2/434 strain, six non-synonymous substitutions were found in the variable domain-I (A91T and T92A) and variable domain-II (N162S, H165Y, L173I and D180Y), where major neutralizing and serotyping antigenic determinants are located [25]. Moreover, several non-synonymous substitutions were selected in more than one case (such as Q75R, D71H and D180Y), suggesting positive selection or recombination events (Fig. 2).

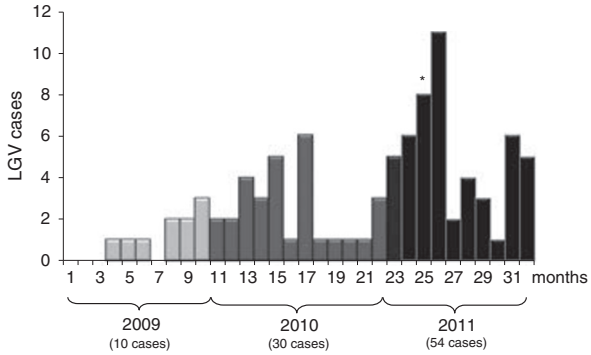

FIG. 1. Temporal distribution of lymphogranuloma venereum (LGV) cases detected during the period between March 2009 and November 2011. The detection of LGV strains was based on the 36 base-pair deletion in the *pmpH* gene according to Schaeffer and Henrich [16]. Moreover, the *pmpH* gene was sequenced in 45 *Chlamydia trachomatis* clinical isolates from LGV and non-LGV samples, confirming in all cases the correct classification of LGV detected. Asterisk shows the date of the first case of LGV in a heterosexual man and also the first cervical sample in our study.

Clinical epidemiological features of LGV cases

As to anatomic site of origin, 419/3185 (13.2%) rectal samples yielded positive *C. trachomatis* amplification, from which 82 (19.2%) were identified as LGV. Ten of 338 (2.6%) positive urethral samples, and two of the 332 (0.6%) positive cervical samples were also LGV genovars; no LGV strains were identified in pharyngeal samples (Table 1). Of the 94 LGV patients, 69 (73.4%) were HIV positive; this percentage would reach 79.3% (69/87) if only MSM were evaluated. Among 51/69 HIV-positive patients with an available viral load test, 80% had a

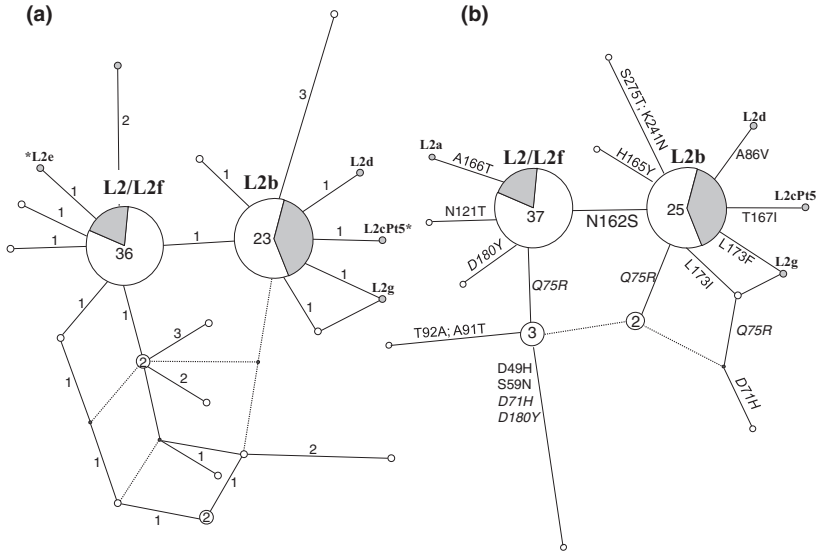

FIG. 2. Phylogenetic network analysis from 75 lymphogranuloma venereum (LGV) strains identified during the period of study based on ompA/major outer membrane protein. Two major nodes are differentiated, corresponding to L2/L2f and L2b variants, which differ in a single amino acid change between them. (a) Network analysis based on nucleotide sequences. Asterisk indicates short sequences. Italics numbers inside the circles indicate the number of strains included in each node and arabic numbers on the lines indicate the number of mutations in each arm. (b) Network analysis using the corresponding amino acid sequences. Amino acidic changes are indicated in each arm referred to L2/434/Bu strain (AM884176). The sequenced fragment was 858 base pairs (starting in the first 20 nucleotides in the leader peptide and ending in the 1106 position). Italics changes indicate common changes to more than one variant. The L2e and L2 sequences are not represented because the fragment used in the analysis is identical to L2f. The numerical differences observed in L2f and L2b nodes between nucleotide and amino acid networks correspond to synonymous mutations. Grey colour corresponds to reference sequences downloaded from GenBank. The filled black dots correspond to non-sampled or extinct ancestral genotypes. The access numbers of reference sequences used in the phylogenetic network were: L2a (AF30485); L2(AM884176); L2b (AM884177); L2c (NC_015744); L2d (EF460797); L2e (EF460798); L2f (EU676181); L2g (EU676180).

TABLE 1. Clinical characteristics of 94 patients with a diagnosis of lymphogranuloma venereum (LGV), according to origin of the samples

Origin	Analysed samples	*C. trachomatis* positive	LGV positive	HIV positive (% detectable viral load)	STI concomitant[a]	Clinical characteristics	Symptoms (%)	Signs (%)
Rectal	3185	419	82[b]	66 (+1 unknown)[c] (40/50 detectable viral load)	42	Purulent discharge	32.9	53.6
						[d]Pain/Tenesmus	60	
						Bleeding	43.9	23.1
						Ulcer	8.5	23.1
						Mucus	4.8	14.6
						Diarrhoea	7.3	
						Itching	8.5	
						None	10.9	
Urethral	2420	388	10[e]	3 (+2 unknown) (1/1 detectable viral load)	5	[f]Purulent discharge	6	
						Ulcer + inguinal lymphadenopathy	2	
						Lymphadenopathy	1	
						Balanitis	1	
						None	0	
Cervical[g]	5462	322	2[h]	1 unknown				
Pharyngeal	2518	100	0					
Total	13585	1239	94	69	47			

C. trachomatis, *Chlamydia trachomatis*; HIV, human immunodeficiency virus; STI, sexually transmitted infection.
[a]STIs, non-HIV, included were syphilis (25 cases), gonorrhoea (13 cases), *C. trachomatis* non-LGV (two cases), genital herpes (one case) and hepatitis B virus (one case).
[b]Only one case was described in a woman.
[c]The viral load was not measured in all patients with HIV.
[d]Symptoms and signs are shown together.
[e]Six patients were men who had sex with men.
[f]Four of these patients presented gonococcal urethritis.
[g]Clinical characteristics are not shown because the number of LGV-positive patients was very low.
[h]One patient was asymptomatic.

detectable HIV viral load, the mean value being 4.86 $\log_{10}$ (Table 1). These worrying data suggest a high risk of co-transmission; in fact, in four patients the diagnosis of HIV was simultaneous to LGV infection. In our series, 50% of the patients also presented other STIs concurrently (Table 1) even though, depending on the location, the prevalence of main pathogens was different. Syphilis (25/82 rectal LGV cases) and gonorrhoea (13/82 cases) were more frequently identified in rectal samples.

Clinical symptoms present in 89.3% (84/94) of patients, ranged between severe proctitis (rectal bleeding) to mild (itching and diarrhoea). Moreover, ten asymptomatic cases were detected in rectal (nine cases) and cervical samples. Those patients carrying the L2b variant presented bleeding and pain more frequently (Table 2) than the group with L2/L2f ($p < 0.009$ and $p < 0.04$, respectively). Furthermore, in general, the patients carrying the L2/L2f variant reported less severe clinical features (or no symptoms) than patients with L2b, except for anal ulcers.

According to all guidelines [13,26], doxycycline (100 mg/12 h for 3 weeks) was prescribed in 75 patients (80%). The criterion for prescription of doxycycline was clinical suspicion of LGV infection (acute symptoms of proctitis). Sixteen individuals without relevant clinical findings or who were asymptomatic were initially treated with a single dose of azithromycin 1 g. In this group, if the genotyping result corresponded to LGV, a second round of conventional treatment with doxycycline was started. In two patients, *C. trachomatis* was eradicated after the initial treatment with azithromycin. Three patients did not receive antibiotic treatment against *C. trachomatis* because it was not suspected and the patients did not return to receive the results.

At the end of the treatment protocol 57/91 (63%) treated patients had their disease controlled, and all except three were negative for *C. trachomatis*. L2b was the variant present in these three patients; they were treated with moxifloxacin (400 mg/day, 7 days) [27]. Finally, in these cases, after moxifloxacin treatment the *C. trachomatis* PCR became negative.

TABLE 2. Clinical characteristics of the patients, depending on the lymphogranuloma venereum (LGV) variants detected

	L2b (%)	L2/L2f (%)	p
Symptoms			
Purulent discharge	20	19.3	ns[c]
Pain	**75**	**45**	**0.035**
Bleeding	**70**	**32.2**	**0.008**
Ulcer	0	9.6	
None	5	12.9	ns
Signs			
Purulent discharge	55	58	ns
Bleeding	30	22.5	ns
Ulcer	25	22.5	ns
Adenopathies	0	9.6	
None	5	6.4	ns
Human immunodeficiency virus status			
Positive	80	87.1	ns
Negative	15	12.9	ns
Unknown	5	0	
Hepatitis C virus status			
Positive	15[b]	12.9	ns
Negative	65	64.5	ns
Unknown	20	22.5	ns
STIs concomitant[a]			
Yes	60	54.8	ns

HIV, human immunodeficiency virus; STI, sexually transmitted infection.
Boldface numbers indicate the symptoms with statically significant differences.
[a]STIs, non-HIV, included were syphilis, gonorrhoea.
[b]Eight HIV-positive patients with LGV in the rectal sample also carried hepatitis C virus.
[c]Not significant.

Discussion

The first LGV case described in Spain was detected in the MSM partner of a man diagnosed in Amsterdam [28]. Since September 2007, new cases of LGV have continued to be diagnosed in the northeast of Spain [10,29,30], with five cases confirmed as belonging to the L2b variant [30]. These data would suggest that these northeast Spanish regions could have been the putative entrance of L2b from the original outbreak in Europe [1,23].

The surveillance and genetic characterization of LGV strains was implemented in Madrid, although no cases had been previously reported, leading to the identification of 94 cases among 13 585 analysed samples. It is worrying that in this period the number of cases continuously increased from 10 to 30 and then to 54 cases. These data reveal an increase of nearly 120% in 2010 and 80% in 2011 with respect to the previous year (Fig. 1). Moreover, in the last year the outbreak extended to women and heterosexual men, indicating the spread towards other population groups. Similar results in two different regions of Spain suggest a wide spread of LGV strains during the period 2008–2011 [10]. In countries with extended surveillance programmes, such as the Netherlands [11] and the UK (www.hpa.org.uk), similar increases were reported. In these countries, as in Spain [10], a bimodal dynamic of the LGV outbreak has occurred. In fact, it is worrying that the accumulation of LGV cases in these countries is higher now than at the beginning of the outbreak. The reason for this increase is unknown. The changes in sexual behaviour, extended sexual networks and immigration flows constitute an ideal setting for the description of new outbreaks and possibly maintenance of this infectious agent in the population if measures such as screening and genotyping programmes are not implemented to avoid re-establishing LGV and prevent long-term complications [31]. These results reinforce the necessity for maintaining the detection of LGV in surveillance programmes.

The most outstanding factor in the LGV outbreak in Madrid has been the identification of two transmission nodes related to two different LGV variants (Fig. 2). A transmission node was constituted by 25 LGV strains belonging to L2b plus five evolved variants derived from it [1,23]. The L2b node was found in a similar proportion between Spaniards and South Americans. A second node included 37 strains with identical *omp*A to an L2/L2f variant (Fig. 2). Moreover, five evolved variants were also described as derived from the L2/L2f-node. This node was found mostly in Spaniards ($p < 0.001$), suggesting a different dynamic of transmission with respect to the L2b node. The L2 strain is more prevalent in America than other regions [32]; in fact the last known outbreak by L2 was in 1992 in the Caribbean area [33]. Considering the high flow of immigrants from South-American countries to Spain between 2005 and 2007 and the well documented South American–Spanish connection, a bidirectional route of transmission of infectious diseases such as HIV [34] and LGV can be expected and we could speculate that L2/L2f arrived in Spain through the South American–Spanish route instead of the L2b European route. These data would suggest that the co-circulation of two LGV variants in Madrid, Spain, could be the result of two independent introductions. It will be interesting to determine if the accumulation of cases observed in other countries such as the UK and the Netherlands, could be related to the presence of L2/L2f in addition to L2b.

On the other hand, the significant association observed between the L2b variant and bleeding ($p < 0.009$) and pain ($p < 0.04$) suggests higher virulence than L2 [35]. On the contrary, the patients carrying L2/L2f presented less aggressive symptomatology, suggesting that those patients with the L2/L2f variant take longer to visit an outpatient clinic, so delaying the diagnosis, and also favouring more transmission events. This evolutionary strategy could provide difficult and late diagnoses and consequently higher transmission rates. This study constitutes a warning for sexual health and public health preventive strategies. Moreover, the high frequency of co-infections, specially in the MSM group, and the high percentage of HIV-positives with a detectable viral load at the same time as LGV diagnosis are suggestive of a very high risk of co-transmissions, in fact in our series four patients were simultaneously diagnosed with LGV and HIV.

In conclusion, to our knowledge this analysis represents one of the most comprehensive studies about the genetic characterization and dynamics of transmission of a worldwide LGV outbreak in a European city characterized by the co-circulation of two LGV variants. LGV attentive surveillance must continue. In this aspect, our study provides a strong contribution for the worldwide picture of LGV molecular epidemiology by providing genetic characterization of strains and through evaluation of LGV transmission dynamics.

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Author Contribution

JdR and JCG conceived and designed the experiments. Laboratory results were obtained by MR, BM, JGA, CR and FJGS. Molecular and phylogenetic analyses were performed by MR, JGA, JCG. TP, TH, PC and MV were responsible for clinical data and follow up and MR, TP, RC and JCG wrote the paper.

Transparency Declaration

The authors declare no conflicts of interest.

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