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Original

Genotyping of *Chlamydia trachomatis* from endocervical specimens of infertile Mexican women

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ABSTRACT

Introduction: It has been reported in several countries that *Chlamydia trachomatis* genotypes D, E, and F are the ones more frequently associated with urogenital infections. In Mexico, the prevalence of serovars and genotypes is unknown.
Material and Methods: One hundred and fifty-two endocervical swabs were collected from infertile women to test for *C. trachomatis*. The PCR-based RFLP and automated-sequencing methods of *ompA* gene was used to identify the *C. trachomatis* genotypes. Sequences of 891 pb obtained were aligned with currently available chlamydial sequences from GenBank to identify the corresponding genotype.
Results: Twenty-four women with infertility (15.8%) were positive for *C. trachomatis*. According to the RFLP and nucleotide sequences results the most prevalent ompA genotype corresponded to serovar F (n = 13 [54.2%]), followed by serovars E (n = 2 [8.7%]), G (n = 2 [8.7%]), K (n = 2 [8.7%]) and LGV (n = 2 [8.7%]), while serovars D, H and Ia were less prevalent (all n = 1 [4.2%]). None of the patients who were positive to genovar L2 had symptoms of lymphogranuloma venereum (LGV). Nucleotide sequences analysis showed a new genovariant of L2, which was different to L2b to L2f. Mutation points were observed in VS1 domain of *Omp A*.
Conclusions: In this study the most common genotypes were F. Furthermore, the L2 genovariants were demonstrated in infertile women without signs and symptoms of LGV disease. Presence of point mutations in L2 genotype sequences were seen by which there is a need for further research in order to identify new L2 genetic variants that exist in Latin America.

Genotipos de *Chlamydia trachomatis* en muestras endocervicales de mujeres mexicanas infértiles

Palabras clave:
Genotipos de *Chlamydia trachomatis*
Infertilidad femenina
Linfogranuloma venéreo
Genotipos L2
Enfermedades de transmisión sexual

RESUMEN

Introducción: En diversos países se ha informado que los genotipos de *Chlamydia trachomatis* más frecuentes y que están asociados a infecciones urogenitales son: D, E y F. En México, la prevalencia de los serotipos y genotipos no se conoce.
Material y métodos: Se obtuvieron ciento cincuenta y dos hisopos de muestras endocervicales de mujeres infértiles para detectar *C. trachomatis*. La identificación de los genotipos de *C. trachomatis* se realizó mediante la técnica de PCR basado en RFLP y en el método automatizado de secuenciación para el gen *ompA*. Para identificar el genotipo, la secuencia de 891 pb obtenida se alineó con secuencias del gen *ompA* de *Chlamydia* disponibles en el GenBank.
Resultados: Veinticuatro mujeres con infertilidad (15,8%) fueron positivas para *C. trachomatis*. De acuerdo con los métodos de RFLP y de secuencición de nucleótidos, el genotipo más frecuente correspondió al

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serotipo F (n = 13 [54,2%]), seguida por el serotipo E (n = 2 [8,7%]), G (n = 2 [8,7%]), K (n = 2 [8,7%]) y L2 (n = 2 [8,7%]), mientras que los serotipos D, H e Ia fueron menos frecuentes (todos, n = 1 [4,2%]). Ninguno de los pacientes con resultado positivo para el serotipo L2 tuvo síntomas de linfogranuloma venéreo (LGV). El análisis de las secuencias nucleotídicas del serotipo L2 mostró una nueva genovariante diferente de la L2b a la L2F. Los puntos de mutación en esta nueva genovariante se observaron en el dominio VS1 del gen *ompA*.

Conclusiones: En este estudio el genotipo más frecuente fue el F. Además, se demostró la presencia del genotipo L2 en mujeres infértiles que no mostraron signos y síntomas de la enfermedad de LGV. Se evidenciaron mutaciones puntuales en las secuencias nucleotídicas del genotipo L2 por lo cual hay necesidad de una mayor investigación para identificar la existencia de nuevas genovariantes L2 en América Latina.

Introduction

Chlamydiae are intracellular bacterial pathogens that cause a spectrum of clinically significant diseases in humans, and Chlamydia infections are highly prevalent sexually transmitted bacterial infections.[1] According to the World Health Organization, 92 million infections with Chlamydiae are detected globally each year.[2] Complications caused by Chlamydial infection are disproportionately suffered by women, 70% of whom are asymptomatic; if they do not receive appropriate treatment they may develop pelvic inflammatory disease (PID) followed by ectopic pregnancy, tubal infertility or chronic pelvic pain and subsequent scarring of the Fallopian tubes.[1,2]

The Major Outer Membrane Protein (MOMP) constitutes approximately 60% of the proteins of the outer membrane of *Chlamydia trachomatis*; it is coded by the *ompA* gene which contains four variable segments or variable domains (VD) separated by five highly conserved segments.[3] Three of these VD segments are exposed surfaces, enabling the serovars to be classified.[3,4] Currently, there are 19 serovars recognized for *C. trachomatis*, all with well-established profiles.[4]

In several countries, sexually transmitted genotypes of *C. trachomatis*- D, E, F, G, H, I, J and K produce cervicitis while L1, L2 and L3 are associated with the illness known as lymphogranuloma venereum (LGV).[1] In developed countries, it has been reported that genotypes D, E, and F are more commonly associated with cervical, vaginal and urethral infections.[5,6] In the majority of Latin American countries the most frequent genotypes are unknown. Brazil and Argentina have reported that these same genotypes are common in patients with endocervical infection.[7,8] In Mexico, the diagnosis of Chlamydial infection is not routine and infection is not notified to the Health Department. However, studies conducted in Mexico regarding the diagnosis of Chlamydial infections have shown infection rates varying between 4% and 28% of sexually mature women.[9–11] This wide range could be due to the different techniques used for the detection of *Chlamydia*, as well as to the different population types. The significance that this pathogen has for the Mexican population seems clear. Although several studies have established the prevalence of Chlamydial infection in Mexico, the prevalence of different serovars and genotypes is unknown. This study describes the prevalence of *C. trachomatis* genotypes detected in infertile women being treated at the National Perinatology Institute.

Table 1
Genotypes of *Chlamydia trachomatis* detected in endocervix from Mexican women with infertility.

Patient	Age	Clinical data[a]	Number of sexual partners	Genotype of *C. trachomatis*	Other microorganisms isolated[b]
2	36	TO	1	K	Gv
3	34	TO	1	F	Negative
6	28	Vaginal infection	1	L2	Negative
12	30	Vaginal infection	1	F	HPV
21	22	TO	1	G	Ca, Uu
24	34	Adherences	2	E	Uu
32	32	OEF	1	F	Gv
42	24	Abort	1	K	Gv, Uu
48	30	Ovarian cyst	1	F	Negative
56	27	Endometriosis, Adherences	1	L2	St B
73	25	Ovarian cyst	1	H	Negative
83	22	OEF	1	Ia	Gv
161	21	Ovarian cyst	2	F	Gv, Uu
180	33	Endometriosis	1	F	Negative
203	29	Endometriosis	2	F	St B
214	26	Vaginal infection	1	F	Negative
232	33	OEF	1	E	St B
241	31	Hydrosalpinx	1	F	St B
269	35	Endometriosis	2	G	Ca
271	30	OEF	1	F	Gv
287	26	OEF	1	F	Gv, Uu
305	20	Endometriosis	2	D	Gv, Ca
473	34	OEF	1	F	Negative
481	29	Adherences	2	F	Tv

[a] TO, tubal occlusion; OEF, ovarian endocrine factor.

[b] Gv, *Gardnerella vaginalis* (with bacterial vaginosis); Uu, *Ureaplasma urealyticum*; Ca, *Candida albicans*; St B, *Streptococcus agalactiae*; Tv, *Trichomonas vaginalis*; HPV, *human papillomavirus*.

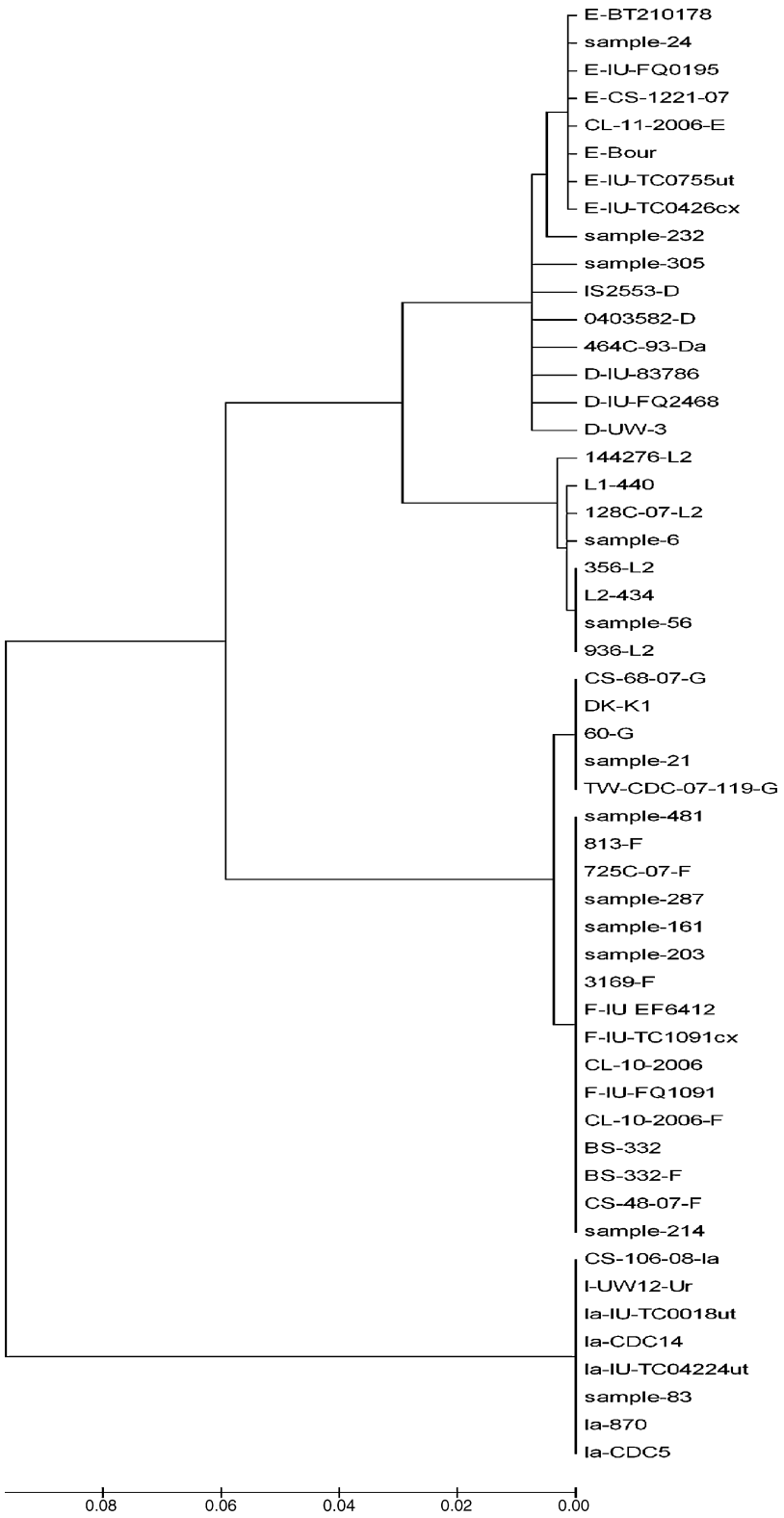

Figure 1. Phylogenetic tree analyses were used to demonstrate the evolutionary relationships between clinical isolates from infertile women and reference strains of *Chlamydia trachomatis* obtained from GenBank. Nucleotide sequences of the *ompA* gene determined in this study were aligned using the MEGA program (version 4).

Materials and methods

Clinical Samples

A total of 152 endocervical swabs were collected from infertile women between the ages of 20 and 35 years who were receiving treatment at the National Perinatology Institute (INPer) and by written informed consent of the use of their endocervical sample for Chlamydial research. The swabs were placed in 2SP medium and then stored at −20 °C until analysis.

DNA extraction

DNA was isolated using a standard protocol employing proteinase K digestion, phenol-chloroform-isoamyl extraction and ethanol precipitation, as was described previously.[12]

PCR of endocervical samples

C. trachomatis was detected by PCR to amplify a sequence of O*mp A* gene which generated a fragment of about 1142 bp. The primers used to amplify *ompA* gene were those reported by Yang et al.[11]; (OMP1 [GCC GCT TTG AGT TCT GCT TCC TC <] and OMP2 [ATT TAC GTG AGC AGC TCT CTC AT]). PCR was performed with 2.7 mM MgCl2, 0.2 mM dNTPs, 30 pM of each primer, 2.5 U of Taq polymerase (GoTaq® Flexi DNA Polymerase Promega©USA) and 5 μl of the DNA sample in a final volume of 25 μl. The reaction mixture was incubated for 5 minutes at 95 °C, followed by 35 cycles of 1 minute at 95 °C for denaturation, 1 minute at 59 °C for annealing, and 1 minute at 70 °C for extension, and a final elongation step of 5 minutes at 70 °C in a thermal cycler (*Programmable Thermal Controller PTC-100 MJResearch©* USA). The secondary PCR reaction contained 2 μl of the initial PCR product, which generated a fragment of 879 bp; the primers used were P3 (T GAC TTT GTT TTC GAC CGT GTT TT) and P4 (TTT TCT AGA TTT CAT CTT GTT CAA T/CTG) also described by Yang et al[13]. Thirty-five amplification cycles were carried out; each consisted of one minute at 95 °C, one minute at 59 °C and one minute at 70 °C. The products from the initial PCR and secondary PCR were visualized by ethidium bromide staining on 2% agarose gel after electrophoresis. *C. trachomatis* serovar L2 (L2 434/Bu) DNA was used as a positive control.

RFLP analysis

Secondary PCR products were submitted to restriction endonuclease *AluI* digestion (Invitrogen) of *ompA* gene for ten hours at 37 °C; 20 μl of reaction mixture contained 2 U of restriction endonuclease *AluI*, 8 μl of PCR products and 2 μl of restriction endonuclease buffer. Restriction profiles were analyzed by 10% polyacrylamide gel electrophoresis at 8 V/cm for two hours.

Sequencing of the ompA gene

The sequencing of the *ompA* gene was carried out on an ABI PRISM 310 genetic analyzer (PE Biosystems) using a BigDye DNA sequencing kit (PE Biosystems) according to the manufacturer's instructions. P3 and P4 primers were used to cover approximately 879 pb of *ompA* gene of *C. trachomatis*.

Results

A set of 152 endocervical samples from infertile women (who had been infertile for approximately three years) were analyzed to investigate *C. trachomatis* infection. Women were aged between 21 and 35 years with a mean age of 27.5. They were all married and reported having an active sex life and did not use any type of contraception method. All patients were asymptomatic to PID or cervical secretions (except for samples 6, 12 and 214). Out of the 152 samples, 24 (15.8%) tested positive for *C. trachomatis* and 128 were negative. The positive samples were subjected to a Nested- PCR (secondary PCR) for *ompA* gene amplification, RFLP analysis and nucleotide sequences. According to the RLFP-PCR results, the most prevalent *ompA* genotype corresponded to serovar F (n = 13 [54.2%]), followed by serovars E (n = 2 [8.7%]), G (n = 2 [8.7%]), K (*n = 2* [8.7%]), and L2 (n = 2 [8.7%]), while serovars D, H, and Ia were less prevalent (all n = 1 [4.5%]) (Table 1). The sequencing of 879 pb amplicon obtained confirmed these serovars (Fig. 1).

Two samples had a similar nucleotide sequence to L2/434 genotype. The 56 sample did not show any point mutation while the 6th sample had two point mutations in variability regions, one of them in vs1 in the 294 nucleotide (A294T), and other in vs4 in the 1012 nucleotide (C1012T). Another point mutation at 388 nucleotide (T388G) was observed, which belonged to the constant region of gene *ompA* (Fig. 2). The amino acid sequences analyses in these mutation points showed changes at Trp60Gly (A294T) and Leu268Phe (C1012T), Fig. 2.

Discussion

PCR-based RFLP analysis or sequencing of the amplified *ompA* gene, which encodes the MOMP, are currently considered to be more sensitive and more specific methods than serotyping for identifying *C. trachomatis* serovars.[6] These technologies have provided valuable and sensitive means for molecular epidemiological analysis to identify high-risk groups and track sexual networks. Differentiation of chlamydia serovars in clinical isolation may be important for a thorough understanding of the pathogenesis and epidemiology of genital Chlamydial infections. The prevalence of *C. trachomatis* serovars has been identified in several countries of the world, with the serovars D (5-48%), D variants, E (22-44%) and F (8-20%) being predominant in urogenital infections, while G (4-7%), Ga, H (<5%), I (6%), I variants, J (5-13%), and K (5-10%), are less common.[5,6,14,15] Sporadically, genital infections with serovars B and Ba also occur.[14,15] In Latin America and the Caribbean, the prevalence of the serovars of *C. trachomatis* in endocervical and urethral infection is rare. A Brazilian study, reported by Lima et al.,[7] described a prevalence of 19% in women who were treated at a public STD clinic and the genotypes associated with endocervical infections in these women were: D (33.3%), E (33.3%), F (16.7%) and K (16.7%). In a recent Argentine study, reported by Gallo et al[8] it reported that most prevalent genotypes in endocervical swabs obtained were, E (46.9%), D (21%) and F (16.1%). In this study, the prevalence was different to the one reported by Lima or Gallo; the most prevalent genotype was F (54.2%), followed by genotype E (8.7%), G (8.7%), K (8.7%), and L2 (8.7%). In this study the most interesting was to identify the L2 genotype in infertile women without signs or symptoms of LGV.

LGV is a sexually transmitted disease caused by serovars L1, L2, and L3 of *C. trachomatis*.[1] Nowadays serovar L2 is the most common cause of proctitis in Europe.[16,17] LGV is endemic in Africa, India, Southeast Asia, South America, and the Caribbean.[18] In this study 2/24 Chlamydia positive samples were L2 genotypes. The Mexican Secretary of Health reported that as of week 51 of year 2009, there had been 144 reported cases of LGV in Mexico, of which 84 corresponded to women and 60 to men.[19] States in the Mexican Republic where women were most affected were: Baja California, Chihuahua, Sonora and Sinaloa.[19] Despite evidence that women are more likely to become infected, transmission routes of LGV have not been considered. There is also a lack of socio-economic data and information on the sexual behavior of women. It is therefore

necessary to conduct more studies in the future so as to enable us to identify the risk factors for acquiring this disease.

The classic picture of LGV usually involves lymph nodes and is characterized by buboes.[17] However, there have been reports of unusual clinical pictures of LGV; for example, the current epidemic is mainly characterized by cases which present severe proctitis. LGV proctitis in men who have sex with men (MSM) is well recognized.[17,20–22] For instance, it is still unclear why inguinal cases were less frequently found in these LGV cases. Other clinical presentations have yet to be studied in more detail since asymptomatic and sub-clinical cases were also identified.[22] Only one case of urethritis due to genovar L2b had been reported so far.[23] However, Gomes *et al*,[24] recently reported the detection of 7 LGV specimens collected from men and women without symptoms or who had no

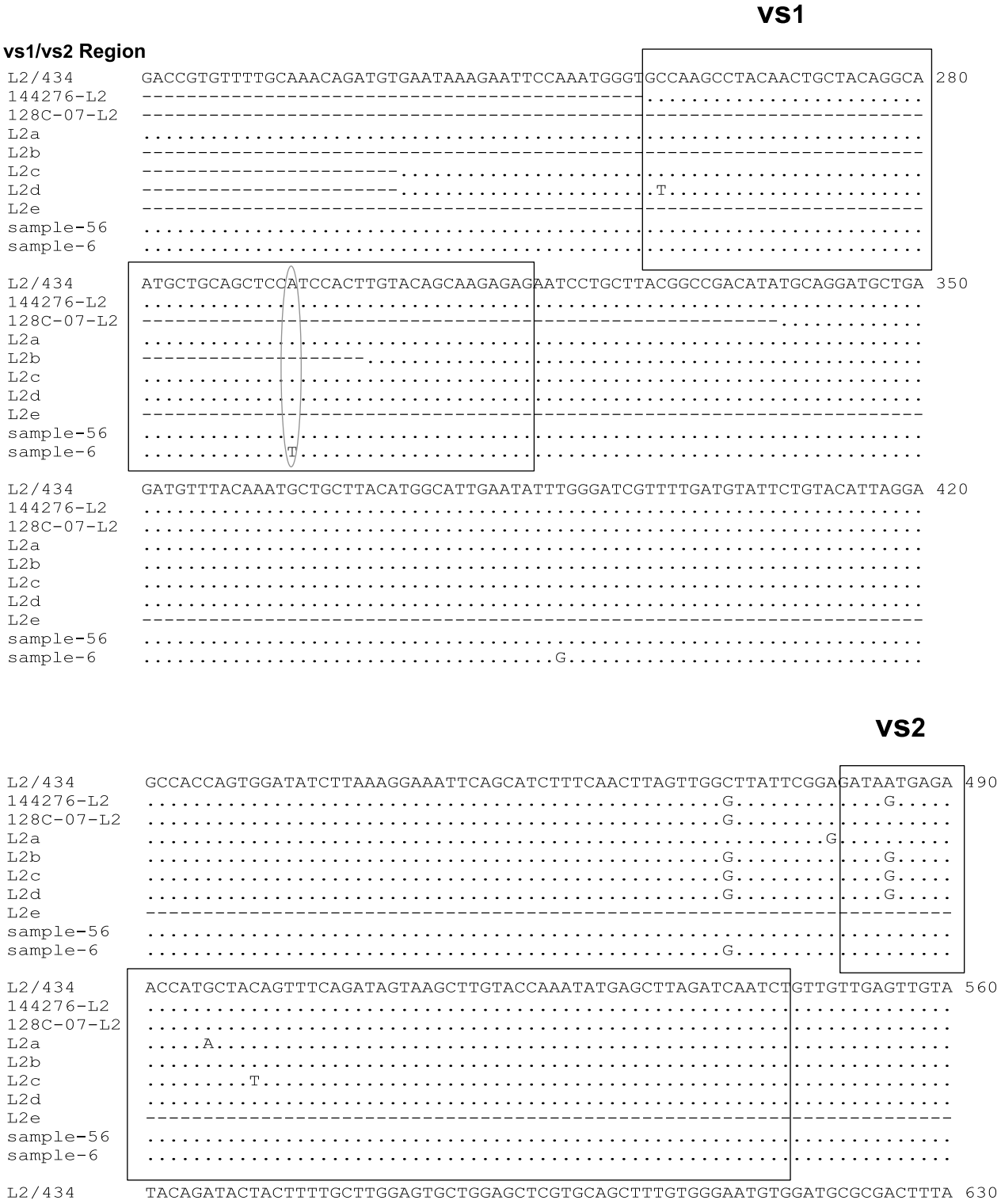

Figure 2. Nucleotide sequence analysis of vs1 and vs4 domains of *ompA* gene from L2 genovariants. Comparison of nucleotide sequences of strain L2/434 (GenBank no. M14738), L2/144276 (GenBank No. DQ217607), L2/128c-07 (GenBank no. EU676181), L2a (GenBank no. AF304858), L2b (GenBank no. AY586530), L2c (GenBank no. EF460796), L2d (GenBank no. EF460797) and L2e (GenBank no. EF460798) with the L2 Mexican variants. The alignment was realized by Clustal W and Bioedit programs (version 7.0.9.0).

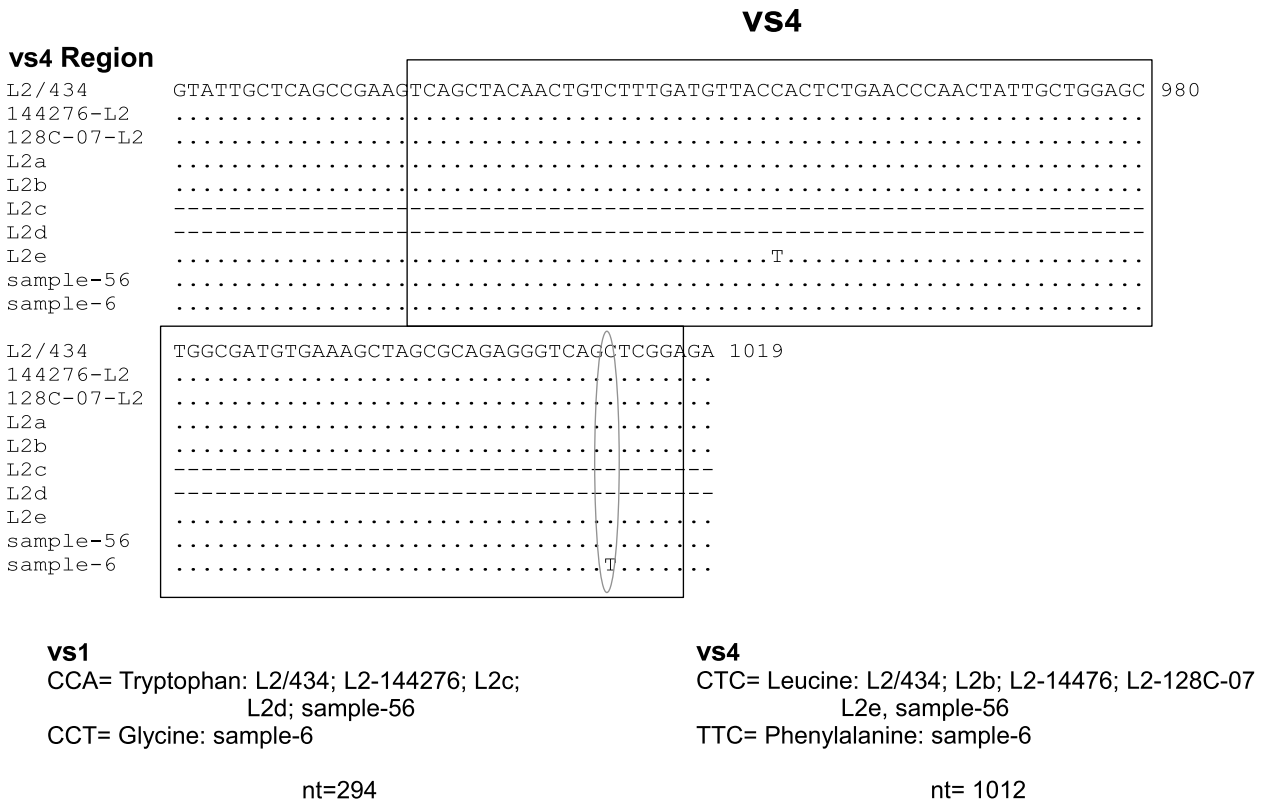

Figure 2. (*Continuación*)

clear LGV symptoms. These samples revealed *ompA* gene sequences different from L2/434 reference strain and from the L2b variant.[24]

New L2 genetic variants have been described recently, designated as L2b, L2c, L2d, L2e, L2f and L2 g and exhibit mutations in the *ompA* gene.[22,24,25] Currently, the L2b to L2e genovariant are associated with the development of proctitis in MSM, while L2f and L2 g are associated with asymptomatic heterosexual men or women.[22,24,25] In this study in the women who were positive to *C. trachomatis* L2 genotype, we analyzed the nucleotide sequence of these variants and compared them with those already reported in GenBank.

The nucleotide sequence analysis in new genovariants (L2a to L2 g) reported that point mutations are observed in VS2 domain[22,24,25] while in this investigation a L2 positive sample (6 strain) showed point mutations in VS1 domain. Stary and et al also describe a point mutation in VS1 in nucleotide 258 (C258T) of L2d genovariant,[25] while in this study a one point mutation was observed in A294T. The difference in these point mutations may be because the LGV strains are isolated in other continents. However, one point mutation in C472G was observed in 6 samples in this study, which is very similar to the one reported in L2b, L2c, L2d, L2f and L2 g genovariants; this suggest that L2 variants could be of a common origin.

Another point mutation has been reported in vs4 domain in L2e variant (C954T). In this study a point mutation in this region was observed in sample 6 in nucleotide 1012 (C1012T).

If these point mutations observed in VS1, VS2 and VS4 domains of several L2 variants provoke changes in amino acid sequence, this could be an indication that this bacterium can carry out the evasion of the immune response of host.

Although these results do not explain fully why patients infected with L2 genotype did not develop LGV symptoms, they might suggest that more than one point mutation may be present in the genome of L2 variants that do not development symptoms of LGV or proctitis. Finally, more research is needed to look for more point mutations in the genome of the new L2 genovariants identified that will enable us to explain why these genovariants do not provoke clinical manifestations of LGV and whether these could or not develop PID, tubal infertility or ectopic pregnancies.

Conflict of interest

The authors have no conflict of interest to declare.

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