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Original Article

Genotyping and prevalence of *Chlamydia trachomatis* infection among women in Belém, Pará, northern Brazil

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Abstract

Introduction: Genital *Chlamydia trachomatis* infection is one of the most prevalent sexually transmitted diseases in women, and undetected cases of the disease are highly associated with long-term complications. Despite the high prevalence of infections in Brazil, very little is known about the distribution of *C. trachomatis* genovars. In this study, we determined the prevalence and genotypes of *C. trachomatis* in women treated at a public hospital in the Brazilian city of Belém, the capital of the state of Pará.

Methodology: A total of 154 women were tested for chlamydial infection by PCR using specific primers for the *C. trachomatis* cryptic plasmid. Genotyping of positive samples was performed by sequencing the *ompA* gene and conducting further phylogenetic analysis.

Results: Out of the 154 samples, 17 were found to be positive using *C. trachomatis* cryptic plasmid PCR. The overall prevalence of *C. trachomatis* infection was 11%, with the highest prevalence observed in women between 16 and 20 years of age. Five genotypes were found to be associated with endocervical infection. Genotype F was most frequently found (37.5%), followed by genotypes J (25%), E (25%), I (6.25%), and D (6.25%).

Conclusions: This study emphasizes the relevance of *C. trachomatis* infection in the young female population of the Brazilian Amazon region. It also demonstrates the diversity of genotypes involved in genital infection in this population.

Key words: *C. trachomatis*; genotyping; *ompA* gene.

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Introduction

Chlamydia trachomatis (CT) is an important human pathogen that causes various diseases and syndromes, including urogenital infection, trachoma and lymphogranuloma venereum, depending on the serovar involved [1]. As an agent of urogenital infection, *C. trachomatis* is the most prevalent bacterial sexually transmitted disease (STD), with estimated 92 million new cases annually worldwide [2]. Approximately 50% of men and 70% of women infected individuals are asymptomatic, making it difficult to diagnose and treat. When the infection remains untreated, complications such as pelvic inflammatory disease may occur, leading to severe sequelae like ectopic pregnancy and infertility [3,4].

Currently, 19 serovars and related variants (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2, L2a and L3) of *C. trachomatis* have been identified by using polyclonal and monoclonal antibodies against the major outer membrane protein (MOMP) [5,6]. Serovars A, B and C have usually been associated with trachoma, whereas D through K have tended to correlate with urogenital infections. Serovars L1 though L3 are commonly associated with lymphogranuloma venereum [7].

Typing of *C. trachomatis* strains remains an important goal in the field of epidemiology, as do clinical and basic research on *C. trachomatis* infections. The temporal and geographical distribution of strains throughout the world has significant implications for our understanding of the epidemiology of this infectious agent and for vaccine development [5]. Moreover, typing can be an important tool to reveal transmission pathways and associations with different tissue tropisms and pathogenicity [8].

The traditional immunotyping methods are currently being replaced by genotyping methods, such as restriction fragment length polymorphism (RFLP) or DNA sequence analyses of the major outer membrane protein (MOMP) gene *ompA* (1) [5,9]. The availability of the whole genome sequence for *C. trachomatis* has led to the development of several new genotyping

systems, such as multilocus sequence typing (MLST) and multilocus variable number tandem repeat analyses (MLVA), with a higher discriminating capacity [10-12]. However, these approaches have been used within the framework of the current *ompA*-based *C. trachomatis* classification system, as there is not yet a standardized nomenclature or classification system based on these novel typing methods [8].

In Brazil, screening for *C. trachomatis* is not routinely available, and it is not mandatory to notify patients that have it. However, isolated studies carried out in this country have shown infection rates ranging from 6% to 31% among women [13,14]. The large range of reported rates reflects discrepancies in both methods used and populations evaluated [15]. Despite a high prevalence of *C. trachomatis* infection in Brazil, very little is known about the distribution and diversity of genotypes involved in genital infection.

In this study, we determined the prevalence and the genotypes of *C. trachomatis* infection in endocervical specimens from 154 women treated in a public hospital in the city of Belém (Pará), the second-largest capital in the Brazilian Amazon region.

Methodology

Clinical specimens and processing

Endocervical samples were obtained from 154 women attending Fundação Santa Casa de Misericórdia do Pará, a public hospital in the city of Belém, Pará, Brazil, between July 2012 and February 2013. The samples were obtained from recipients of three gynecological services offered by the hospital: STD screening, family planning and prenatal care. The ethical clearance for this study was granted by the IEC (CAAE: 02394512.3.0000.0019), on June 18, 2012. All women were informed and gave their written consent to participate in the study, and completed a clinical and epidemiological questionnaire. The specimens were collected with Dracon-tipped swabs or brush during routine pelvic examinations, and were subsequently placed into sterile collection tubes containing 3 ml of specimen transport medium (DIGENE). After being vigorously vortexed within the collection tubes, the swabs were removed. For pregnant women, only cervical swabs were obtained. The samples were sent to the Molecular Biology Lab at the Evandro Chagas Institute, and stored at -20º C to await further analysis.

Screening of C. trachomatis

The endocervical samples were submitted to DNA extraction using the PureLink Genomic DNA kit (Invitrogen, Carlsbad, USA) as the manufacturer directs. To verify the efficacy of DNA extraction and the presence of PCR inhibitors, the samples were first screened using PCR specific primers for the human β-globin gene. In order to detect *C. trachomatis* DNA in the samples, a 241 base pair (bp) fragment of the genetically conserved cryptic plasmid of *C. trachomatis* was amplified using PCR [16], and *C. trachomatis* serovar L2 DNA was used as a positive control. The amplified products were visualized on a 1% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, CA). The results have been confirmed by using Hybrid Capture HC II CT-ID DNA Kit v 2.0 (DIGENE, Gaithersburg, Maryland, USA).

C. trachomatis genotyping

C. trachomatis PCR-positive samples were selected for genotyping by amplifying a 990 pb fragment of the *ompA* gene according to a nested-PCR methodology (Table 1) described elsewhere [17].

The *ompA* fragments obtained were purified using the QIA Quick PCR Purification Kit (Qiagen, Santa Clarita, CA, USA) and bidirectionally sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) using two inner primers (MOMP87 and RVS1059) and a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Genotyping was performed based on a BLAST similarity search, and a phylogenetic tree analysis was generated to illustrate the evolutionary relationships between clinical isolates and reference strains. Using version 7.0 of the software program BioEdit, each sequence obtained was aligned with an analogous sequence from reference strains. The strains were derived from a GenBank database: (Gen bank accession number M58938, AF063208, M17343, X62918,

Table 1. Primer sequences used for *C. trachomatis* plasmid PCR and *ompA* gene PCR.

Primer	Target	Reference	Strand	Sequence (5'-3')
KL1	Cryptic plasmid	Mahony *et al*., 1995	sense	TCC GGA GCG AGT TACG AAG A
KL2			antisense	AAT CAA TGC CCG GGA TTG GT
P1	*ompA* gene	Lysen *et al.,* 2004	Outer sense	ATGAAAAAACTCTTGAAATCG
OMP 2			Outer antisense	CTCAACTGTAACTGCGTATTT
MOMP87			Inner sense	TGAACCAAGCCTTATGATCGACGGA
RVS			Inner antisense	TCTTCGAYTTTAGGTTTAGATTGA

X62920, X52557, X52080, AF063199, X16007, AF063200, AF063201, AF063202, AF063203, AF063204, M36533, M14738 and X55700. Strains of *Chlamydia muridarum* MoPn (M64171) were used to constitute an outgroup. Phylogenetic trees were generated using the maximum-likelihood method implemented in the MEGA software program (version 6.0; Tamura, Dudley, Nei, and Kumar [http://www.megasoftware.net/]). Finally, a confidence level was estimated using bootstrap resampling on 1,000 randomly selected pseudoreplicates.

Statistical analysis

Statistical analysis was performed using version 5.0 of the software program BioEstat [18]. Association of chlamydial genovars with demographic and behavioral characteristics was assessed using the chi-square or Fisher exact tests. Statistical significance was established as $p < 0.05$.

Results

Studied population and C. trachomatis prevalence

A total of 154 women participated in the study: 72 through the family planning service, 64 through the STD-screening service, and 18 through the prenatal care service. The age of patients ranged from 12 to 46 years; their mean age was 24.3 years. All endocervical specimens were successfully screened using β-globin PCR and no inhibition was detected. Of the 154 samples, 17 (11%) were found to be positive using *C. trachomatis* cryptic plasmid PCR and Hybrid Capture HC II CT-ID DNA Kit v 2.0 (DIGENE). When only the group of women under 20 years of age was analyzed, the prevalence increased to 20.4% (Table 2), and a significant association was observed between age and the prevalence of *C. trachomatis* infection (p = 0,041). After comparing the prevalence among each group that received hospital services, the highest prevalence of *C. trachomatis* was observed among patients who had specifically sought STD screening services (15.6%), followed by those who sought prenatal care (11.1%) and those who sought family planning (6.9%), although the difference in rates of infection was not significant (Table 3).

Figure 1. Plylogenetic tree for *ompA* gene nucleotide sequences. The tree was generated using a maximum likelihood method. Booststrap values for 1000 pseudoreplicates.

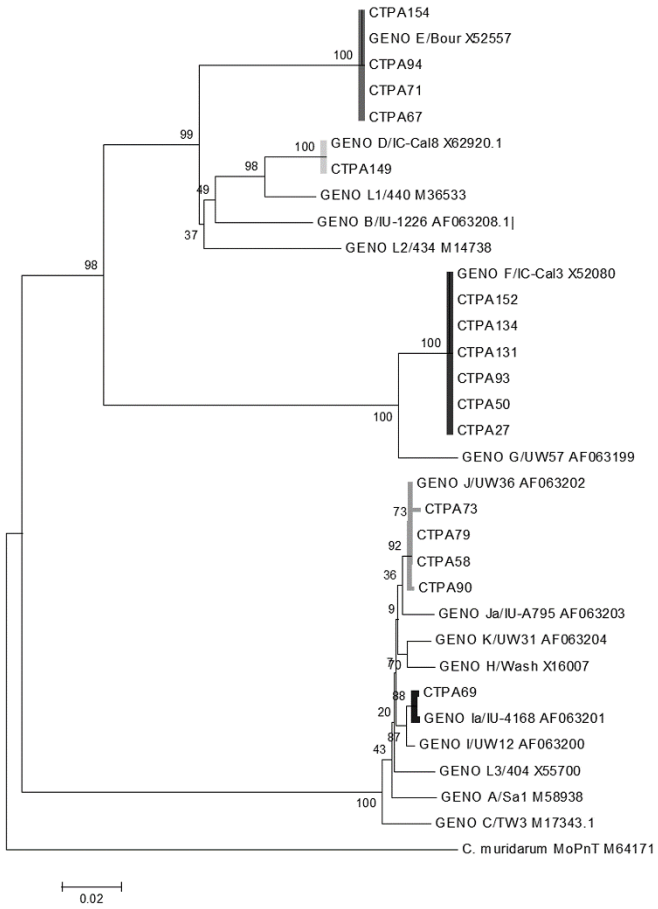

Table 2. Positive results for *C. trachomatis* in relation to age.

Age group (years)	Numbers of patients (%)	*C. trachomatis* PCR-positive (%)	p-Value*
< 20	54 (35.0)	11 (20.4)	0,041
20-30	62 (40.3)	5 (8.1)	
> 30	38 (24.7)	1 (2.6)	
Total	**154 (100.0)**	**17 (11.0)**	

* Chi-square test

Table 3. Positive results for *C. trachomatis* in relation to gynecology service.

Source service of patients	Numbers of patients (%)	*C. trachomatis* PCR-positive (%)	p-Value*
STD screening	64 (41.5)	10 (15.6)	0,354
Family planning	72 (46.8)	5 (6.9)	
Prenatal care	18 (11.7)	2 (11.1)	
Total	154 (100.0)	17 (11.0)	

* Chi-square test

Genotyping Analysis

All 17 positive clinical samples for the cryptic plasmid PCR were subjected to a nested PCR for *ompA* gene amplification. The 16 samples that were successfully amplified generated a fragment of approximately 900 bp and contained all four variable domains of the *ompA* gene.

Sequence analysis of the *ompA* gene revealed that the F genotype was the most prevalent (n = 6; 37.5%), followed by E (n = 4; 25%), J (n = 4; 25%), I (n = 1; 6.25%) and D (n = 1; 6.25%). The phylogenetic analysis produced a tree that grouped the analyzed samples into five clades. Bootstrap values ranging from 86% to 100%, confirmed the identification of the five different genotypes of *Chlamydia trachomatis* in the sample studied (Figure 1).

ompA sequences obtained in our sample showed high similarity values, ranging from 99.76 to 100% when compared to those from the literature. Genotype F was highly conserved, showing 100% similarity between the sample sequences and the reference sequences, whereas genotype sequences D, E, J and I displayed either one or two nucleotide substitutions when compared to the reference sequences (Table 4). In all, nine nucleotide substitutions were observed, eight of which resulted in amino-acid replacement.

Both patient age and clinical signs and symptoms (lower abdominal pain, abnormal vaginal discharge, ectopia and cervicitis) were analyzed for possible associations with particular *C. trachomatis* genovars, but no statistically significant associations were found.

Discussion

Studies performed in several countries show that *C. trachomatis* is the main bacterial agent of sexually transmitted disease worldwide. Variable rates of infection prevalence have been found, depending on which populations were studied and which diagnostic methods were used. Though the tracking of *C. trachomatis* is not mandatory in Brazil, several studies of prevalence have been performed in different regions of the country, with rates that have varied from 6% to 31% [13,14]. In the Amazon region, these studies are scarcer. Data mainly from female patients at an STD clinic in the city of Manaus indicated a *C. trachomatis* prevalence of 13% [19]. There are, however, no data about the genotypes that were involved in these infections.

In the present study, the prevalence of *C. trachomatis* and its genotypes was analyzed in a sampling of women from Belém, Pará, the second-largest capital in the Brazilian Amazon region. The global prevalence of chlamydial infection (11%) was similar to that found in other studies performed in Brazil under similar conditions, reflecting a national reality [20]. In 2008, Jalil *et al*. performed a wide-ranging, multicentric study in the capitals of six Brazilian states. In a population of approximately 3,000 pregnant

Table 4. Nucleotide changes found in *ompA* gene of *C. trachomatis* compared to reference sequences[a].

Sample Identification	GenBank accession no.	Genotype	Similarity (%)	Nucleotide Change	Position[b]	Amino acid change
CTPA 27	KP269164	F	100.00	-	-	-
CTPA 50	KP269165	F	100.00	-	-	-
CTPA 93	KP269166	F	100.00	-	-	-
CTPA 131	KP269167	F	100.00	-	-	-
CTPA 134	KP269168	F	100.00	-	-	-
CTPA 152	KP269169	F	100.00	-	-	-
CTPA 67	KP269170	E	99.88	G to A	997	A to T
CTPA 71	KP269171	E	99.76	A to G C to T	194 1034	Y to C S to F
CTPA 94	KP269172	E	100.00	-	-	-
CTPA 154	KP269173	E	100.00	-	-	-
CTPA 58	KP269174	J	100.00	-	-	-
CTPA 73	KP269176	J	99.76	T to G A to G	625 746	C to G E to G
CTPA 79	KP269177	J	100.00	-	-	-
CTPA 90	KP269178	J	98.88	G to A	272	S to N
CTPA 149	KP269179	D	99.77	G to A	1013	A to T
CTPA 69	KP269175	I	99.76	A to G G to A	526 1050	I to V Silent (L)

[a] The following reference strains were used for comparison with sequences obtained in this study: F/ICCal3 (X52080), E/Bour (X52557), J/UW36 (AF063202), D/IC-Cal8 (X62920) and Ia/IU-4168 (AF063201); [b] Position refer to reference sequences of each genotype and are not homologous between serotypes.

women, a *C. trachomatis* prevalence of 13.4% was observed [21].

In accordance with the findings of other studies, our results also showed an association between *C. trachomatis* and patient age, with the prevalence among women under 20 amounting to 20.4%. This rate, one of the highest ever described in Brazil, confirms youthful age as an important risk factor for *C. trachomatis* infection [19].

Although a large body of work demonstrates the high prevalence of CT infection in the Brazilian population, only two studies describe the distribution of urogenital *C. trachomatis* genotypes in the midwestern and southeastern regions of the country [15,22]. To our knowledge, this is the first study to describe the genotypic diversity of *C. trachomatis* associated with genital infection within a population residing in the northern of the country. Five different genotypes were identified in endocervical samples, with F, E and J appearing most frequently. The D genotype was detected in only one case.

The first study undertaken for genotyping of *C. trachomatis* in Brazil analyzed 12 sequences of the *ompA* gene in women treated at an STD clinic in the midwestern city of Minas Gerais. It found four genotypes. At 33.3% each, E and D were the most frequently detected, followed by F and K, which each showed a frequency of 16.7% [15] A broader study by Machado *et al*. (2011) genotyped 163 positive *C. trachomatis* samples from men and women in two regions of Brazil: the midwestern city of Goiania and the southeastern city of Vitoria. In this study, nine distinct genotypes were encountered, the five most common being E (39.3%), F (16.6%), D (15.9%), I (8.6) and J (7.4%) [22]. In both studies, genotypes E, D and F were identified most frequently in samples from subjects with urogenital infections of *C. trachomatis,* respectively comprising 83.3% and 71.8%, of the genotypes found. These results accord with the findings of studies in other countries (such as Argentina, Costa Rica, Taiwan, Iran and Hungary), in which the same three genotypes were found to appear most frequently in urogenital cases of *C. trachomatis* [6,7,23]. In the present study, genotype F was the most frequent, followed by genotypes D and J. These findings revealed differences between the genotypic distribution of urogenital *C. trachomatis* in women from the Amazon region and that of women elsewhere in Brazil and the world. However, since the sample size is limited, more studies are warranted to better understand the distribution of *C. trachomatis* genotypes in this population.

Conclusions

The high prevalence of chlamydial infection, especially among young women, reinforces the importance of public STD-prevention policies for the aforementioned age group. More studies are necessary to better understand the epidemiology and distribution of *C. trachomatis* genotypes in women from the Amazon region.

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