Chlamydia trachomatis infection and distribution of serovars in the Eastern Highlands Province, Papua New Guinea

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SUMMARY

We have used nested polymerase chain reaction (PCR) and the PCR-based endonuclease digestion method to genotype Chlamydia trachomatis serovars in 460 infected individuals from the Eastern Highlands Province of Papua New Guinea. Our study groups comprised women who presented in labour to the Goroka Base Hospital, their newborn infants, symptomatic children who presented to the hospital’s Outpatients Department and men and women from 15 randomly selected villages in the Asaro Valley. In this analysis, the major outer membrane protein (MOMP) gene, omp1, of C. trachomatis was amplified using DNA obtained from the endocervix of women, urine from men, and both the eye and nasopharynx of children. Amplified DNAs were digested concurrently using AluI and a combination of EcoRI, HinfI and HpaII restriction enzymes. The mixtures were separated on electrophoretic gels and the respective serovars designated on the basis of resolved digested DNA patterns. Our results, which were confirmed also by omp1 sequence data, show serovars D, E, F, G, H and L3 to be present in the studied communities. The overall relative frequencies of these serovars were 30%, 21%, 25%, 1%, 20% and 2% respectively, with serovars D, E, F and H accounting for 97% of these infections. Double infections among these principal serovars were also detected in all our study groups but at a low overall frequency of 3%. Serovar D was the major agent involved in the aetiology of chlamydial infection in both children and adults though serovar F was the most frequent in newborn infants. Serovar H was relatively less frequent in symptomatic children. No trachoma-related serovars were detected, confirming the rarity of this disease in Papua New Guinea. In contrast, although clinical cases of lymphogranuloma venereum have not been described in the country, the detection of serovar L3 in this study suggests that it may occur. However, the association
of L3 also with childhood infection indicates that it may be causing the same pathology as the serovars D-K that are associated with non-ulcerative sexually transmitted infections.

Introduction

Chlamydia trachomatis is a major cause of the severe morbidity resulting from sexually transmitted infections (STIs) worldwide (1). It has been recognized as the leading cause of bacterial STIs in the United States (2) and other developed countries (3,4). The prevalence and impact on disease burden attributed to this pathogen is expected to be greater in resource-poor settings, such as among ethnic minorities (5,6) and developing countries (7), given their high background of STIs.

This pathogen presents a spectrum of clinical disease that is similar to that caused by gonococci in infected hosts. However, unlike the more acute disease from the more highly transmissible Neisseria gonorrhoeae, transmission of C. trachomatis occurs at an observably low rate (8) and symptoms when present are less apparent. Ironically, due to its reported chronicity and fulminating character (5,9,10), progressive infections by this organism in both the ocular and genitourinary tract can result in life-threatening conditions. This appears to be immunologically mediated. The immunopathological responses elicited against the chlamydia heat-shock protein (11,12) and other antigens, in particular the major outer membrane protein (MOMP) (12-15), cause scarring and pathological changes in the superficial epithelia and columnar cells leading to disease. In infected women, the subsequent tissue destruction, fibrosis and scarring of the columnar endothelial linings in the upper genitourinary tract can result in chronic abdominal pain, pelvic inflammatory disease (PID) and ectopic pregnancies (16). Both female and male adults may become sterile following occlusion of the reproductive tract (12) and children born to infected women can develop neonatal conjunctivitis and pneumonia (17), with the latter group being predisposed to pulmonary complications later in life (18). The potential of this happening is said to increase over time with persisting and recurrent infections (12,15). Moreover, recurrent infections can also result in reactive arthritis and, in pregnant women, can lead to premature rupture of the amniotic membranes and preterm births (19,20).

Disease-causing strains of C. trachomatis have been broadly categorized into 18 major serological variants or serovars, now determined by genotyping of the major outer membrane protein gene. These include serovars A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, K, L1, L2, L2a and L3. Traditionally, serovars A to C have been associated with trachoma, serovars D to K with non-ulcerative STIs and serovars L1 to L3 with lymphogranuloma venereum (LGV). Whether or not pathogenicity varies between serovars is not clearly established because it is usually difficult to grow this organism for such analysis. However, what is known is that both T-cell and humoral responses elicited against chlamydial infection appear to be serovar specific (21), and therefore not broadly protective. This suggests that reinfection with a different serovar may enhance hypersensitive immunity thereby contributing to progressive disease.

Preferential serovar infection in certain hosts and diseases (22) and association of some serovars with specific clinical manifestations (23), or their influence in confounding clinical diagnosis in heterogeneous STI infections (24), further support the concept of serovar-specific disease associations. New insights into this field show, however, that this is not always the case. The detection of trachoma-related variants, serovars B and Ba, in the genitourinary tract (25-27) is a case in point. Indeed, both serological relatedness studies and recent nucleotide and deduced amino acid analyses (28) define these serovars as belonging to the B serocomplex, which also includes serovars D, E, L1 and L2. The other agent of LGV, serovar L3, is found to be more related to the C serocomplex group which comprises serovars A, C, H, I, J and K, with serovars F and G making up the third serogroup. Regional variation is another factor to be considered and apparent disease associations with some serovars may really be a consequence of their geographic
Studies to date have shown *C. trachomatis* to be highly prevalent in Papua New Guinea (PNG) (29-33). However, no data have been available about which serovars are prevalent in the country. The identification of *C. trachomatis* serovars constitutes an essential step towards greater understanding of the epidemiology of chlamydial infections. While the detection of chlamydial STIs implies that the relevant serovars are present, no epidemiological or clinical data are available on trachoma-related serovars in the country. Both trachoma and LGV have been assumed to be rare, or even non-existent, but this has not been rigorously documented. As a first step in determining the distribution of *C. trachomatis* serovars in PNG, we have tested previously collected samples in the Eastern Highlands Province (EHP) to establish the serovars found in the study population groups there.

### Materials and Methods

#### Study populations

Samples that were determined previously to be positive for *C. trachomatis* by direct fluorescent antibody test (DFA) (Behring Diagnostics) and polymerase chain reaction (PCR) were used. The samples represented a cross-section of population groups studied in 1991 and between June 1994 and March 1998. They comprised first-void urine from men and endocervical swabs from women living in 15 randomly selected villages of the Asaro Valley in EHP (30, 31), endocervical swabs from women who presented in their first stage of labour for delivery at the Goroka Base Hospital (GBH) and both eye swabs and nasopharyngeal aspirates from the newborn infants of these women (32). Eye swabs collected previously from children who presented with eye discharge to the Outpatients Department of GBH (33) were also tested. Ethical approval for these studies was obtained from the Medical Research Advisory Committee of Papua New Guinea.

#### Polymerase chain reaction detection

A 1200 base-pair (bp) gene segment, *omp1*, spanning almost the entire region of *C. trachomatis* MOMP, was amplified from crudely extracted DNA using forward primer FLS (5'-CTC TTG AAA TCG GTA TTA GTA TTT GCC GCT-3') and reverse primer FLA (5'-TTA GAA GCG GAA TTG TGC ATT TAC GTG AGC-3') (34). PCR was done in a volume of 50 µl using 5 µl of the crude DNA lysate, 50 mM KCl, 50 mM Tris (pH 9.0), 1.5 mM MgCl$_2$, 0.1% Triton X-100, 0.2 mM dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer and 0.04 units of Taq DNA polymerase. Samples were subjected to 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, with final extension at 72°C for 7 minutes. A 1 µl aliquot of this primary PCR product was reamplified using species-specific internal primers CT7 (5'-TGA CTT TGT TTT CGA CCG TGT TTT-3') and CT6 (5'-TTT TCT AGA TTT CAT CTT GTT CAA T(C)TG-3') as previously described by Gaydos and co-workers (35). Amplified products were resolved by electrophoresis on 1% agarose gel and stained with 0.01 µl/ml ethidium bromide solution for confirmation.

#### Restriction endonuclease digestion

Ten microlitre aliquots of CT7/CT6 PCR products were digested concurrently with AluI and a combination of EcoRI, HinfI and HpaII (Promega) restriction endonucleases. Samples were incubated at 37°C overnight for 16 hours in appropriate restriction buffers. 8 microlitres of the digested DNA mixtures were resolved separately on 12% polyacrylamide gels at 10.5 volts/cm for 2.5 hours at room temperature. The restricted DNA fragments were detected by silver staining.

#### Sequencing

The dideoxy chain termination method was performed independently to confirm the initial results of *C. trachomatis* genotypes obtained by endonuclease digestion. PCR products of primers FL8/FL3 and N2/N4 (M. Ward et al., Southampton University, UK), which span the variable segments II and IV of *omp1* respectively, were used as templates for this analysis.

### Data analysis

Data analysis was carried out using Stata 8.0 (Stata Corporation) and Epi Info 6.04b (Centers for Disease Control and Prevention, USA). Associations of serovars and their variables were analyzed using contingency tables and their statistical significance
determined using the Pearson chi-squared test. Where appropriate, the Mantel-Haenszel chi-squared test was performed to test for interaction with possible cofactors.

**Results**

Samples from 460 individuals were successfully tested, including 195 women who presented in labour at Goroka Base Hospital and were subsequently delivered (median age of 23 years), 113 of their newborn infants (median age of 56 days) and 121 individuals from the 15 villages in the Asaro Valley, of whom 19 were men (median age of 26.5 years) and 102 women (median age of 24 years). The majority of the women who presented to the hospital for delivery came from the Asaro Valley but not necessarily from the same villages as the community-based women recruited there. Results were also available for 31 children (median age of 13 days) who presented previously with eye discharges at the same hospital.

Serovars D, E, F, G, H and L3 were detected in the study population groups. Serovars D, E, F and H were the predominant types detected and accounted for 97% of infections overall. Their respective frequencies were 30%, 21%, 25% and 20% (Table 1). Serovars L3 and G were consistently detected at low relative frequencies in all the study groups, accounting overall for 2% and 1% respectively.

The respective distributions for the studied population groups were, however, observed to be variable (Table 1). Except among the newborn infants and the village-based men, serovar D was the most common serovar detected: 27% in the women who presented in labour, 35% in the symptomatic children and 40% in the women recruited from the villages. Serovar E was the commonest type detected in the village-based men and serovar F in the newborn infants, accounting for 42% and 39% respectively. Serovar F was also determined to be the second most common type detected in the women in labour and the village-based men, with relative frequencies of 26% and 32% respectively.

Only 8% of women in the general population were found to be infected with serovar F while the relative frequencies were higher in the infants and the mothers (39% and 26% respectively). The observed difference in the distribution of serovar F between the two groups of women was significant, even when corrected for the year of study ($\chi^2 = 13.34, 1\text{df}, p < 0.001$); our results showed that *C. trachomatis*-positive pregnant women were significantly more likely to be

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Women in labour N=195</th>
<th>Women in labour %</th>
<th>Newborn infants N=113</th>
<th>Newborn infants %</th>
<th>Children with eye discharge N=31</th>
<th>Children with eye discharge %</th>
<th>Village-based women N=102</th>
<th>Village-based women %</th>
<th>Village-based men N=19</th>
<th>Village-based men %</th>
<th>Overall N=460</th>
<th>Overall %</th>
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<tr>
<td>D</td>
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<td>26.5</td>
<td>35.5</td>
<td>40.2</td>
<td>26.3</td>
<td>30.4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>21.0</td>
<td>14.2</td>
<td>32.3</td>
<td>22.5</td>
<td>42.1</td>
<td>21.4</td>
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<td>38.9</td>
<td>16.2</td>
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<td></td>
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</tr>
<tr>
<td>G</td>
<td>1.0</td>
<td>0.9</td>
<td>0.0</td>
<td>2.0</td>
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<td>1.1</td>
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</tr>
<tr>
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<td>17.7</td>
<td>6.5</td>
<td>27.5</td>
<td>0.0</td>
<td>20.3</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>L3</td>
<td>2.6</td>
<td>1.8</td>
<td>9.7</td>
<td>0.0</td>
<td>0.0</td>
<td>2.2</td>
<td></td>
<td></td>
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</tbody>
</table>

**TABLE 1**

**DISTRIBUTION OF CHLAMYDIA TRACHOMATIS SEROVARS DETECTED IN THE STUDIED POPULATION GROUPS**
infected with serovar F than women in the general population who were *C. trachomatis* positive (odds ratio = 5.0, 95% confidence interval = 1.81–14.29, p <0.001). The proportion of pregnant women infected with serovar F also increased significantly with age (by age group <20 years, 20-25 years and >25 years) (χ² = 11.59, 2df, p = 0.003).

Among newborn infants, of the total of 77 infections in the eye, 9 were caused by serovar H and 1 by serovar L3. Similarly, of 96 infections in the nasopharynx, 19 were caused by serovar H and 2 by serovar L3. There was no significant difference in the relative frequency of serovars with respect to samples taken from the eye and the nasopharynx in this group of newborn infants. Serovar L3 was detected at an unexpectedly higher frequency in the children who presented with eye discharges (10%), contrary to its low frequency rates observed in the other groups (Table 1).

Double serovar infections were detected in the population groups studied but at a low frequency rate of 3% overall. 4 individuals in the study groups had mixed infection with serovars DF, 3 with serovars DH, 2 with serovars EF and 1 individual each with mixed serovars DE, EH and FH.

Distributions of the predominant serovars detected in the women who were seen at the hospital during the study period are shown in Figure 1. Except in 1995 where all the four major serovars showed rates between 20% and 30%, their respective rates were markedly variable in the study period. Significant differences over the study period per serovar were noted only in the distribution of serovar E (χ² = 10.24, 4df, p = 0.04) and serovar F (χ² = 9.39, 4df, p = 0.05). All the community-based women were enrolled in 1995. The corresponding frequencies of the respective serovars in the latter group are reported in Table 1.

The restricted DNA fragments generated by the panel of restriction enzymes that were used in the study showed a typical DNA profile for each serovar (Figures 2 and 3). In all cases, serovars D, E, F and G, represented respectively in lanes 2, 3, 4 and 5 of Figure 2,
were readily distinguishable by Alul digestion. Serovars H and L3 (lanes 6 and 10 of Figure 2) are known to share the same restriction site for this enzyme as serovars A, C, I and J (35), and were detected separately by multiple digestions with EcoRI, HindIII and HpaII (Figure 3). Potential variants of serovars H and L3 are also shown in lanes 9 and 7 respectively of Figure 2. The multiple endonuclease restriction patterns for these variants were also noted to be distinct as depicted in Figure 3. Minor shifts in restricted DNA bands also suggested that variants of serovars D and E were present (results not shown). In all these cases, the restricted DNA fragments of multiple digestions maintained the variability of their respective prototype bands. One unresolved profile suggested an infection with more than two serovars to be present, or alternatively the presence of a new variant, but this could not be confirmed due to unsuccessful reamplification attempts. Our RFLP (restriction fragment length polymorphism) genotype results were found to be consistent overall with the sequencing data (Table 2). No sequencing data were available for serovars G and L3 but the restricted DNA banding profile obtained for these serovars in the study appeared to be consistent with previously published patterns (35).

The PCR-based genotyping adopted in this study was found to be efficient in designating the serovars that are present in the studied population groups. However, although amplification of C. trachomatis with primers CT7/CT6 had been shown to be successful in the study of Gaydos et al. (35), some positive samples determined previously by C. trachomatis species-specific PCR with N2/N4 primers in our laboratory failed to amplify in the current study. This failure continued despite several reamplification attempts using

Figure 2. Restricted DNA patterns of C. trachomatis serovars using Alul restriction endonuclease. Lane 1, 1 kilobase (kb) DNA ladder; lane 2, serovar D; lane 3, serovar E; lane 4, serovar F; lane 5, serovar G; lane 6, serovar H; lane 7, variant of serovar L3; lane 8, 1 kb DNA ladder; lane 9, variant of serovar H; lane 10, serovar L3.
Figure 3. Restricted DNA patterns of *C. trachomatis* serovars using EcoRI, HindI and HpaI restriction endonucleases.

Lane 1, 1 kb DNA ladder; lane 2, serovar D; lane 3, serovar E; lane 4, serovar F; lane 5, serovar G; lane 6, serovar H; lane 7, variant of serovar L3; lane 8, 1 kb DNA ladder; lane 9, variant of serovar H; lane 10, serovar L3.

Discussion

This is the first report of serovars of *C. trachomatis* identified from Papua New Guineans, including both asymptomatic and symptomatic individuals. Serovars D, E, F and H were the predominant types found among children and adults in our study communities. Serovar D was the most common type detected in the study groups, except among the newborn infants and the village men, where serovars F and E predominated (Table 1). Serovars D, E and F accounted for 76% of chlamydial infections in the studied groups, slightly more than the 60-70% reported previously among urogenital chlamydia in The Netherlands and Sweden (25,36), and also elsewhere (3,37-40). They appear to be the clinically important serovars in the study population as suggested by the 84% (26/31) carriage rate detected among the symptomatic children.

The high relative frequency of serovar H determined in our study is exceptional as much lower frequencies of this serovar have been reported in the earlier mentioned studies (3, 25, 36-40). Nevertheless, the observed frequencies in the present study are consistent with a high urogenital prevalence that has been reported twice before: among STI patients in The Netherlands and also from individuals who presented to sexually transmitted disease and other related adult consultation clinics in Lisbon (26, 41). In Lisbon, women who were over 25 years old had a significantly higher prevalence of serovar H than younger women or men. Results of the present study show that serovar H was not found in men though it was frequently detected in women and children (Table 1). It was found to colonize both the eye and the nasopharynx in children. A lower frequency noted among symptomatic children shows, however, that it might not be as


**TABLE 2**

**CHLAMYDIA TRACHOMATIS SEROVARs DETERMINED BY RESTRICTION ENDONUCLEASE DIGESTION AND SEQUENCING ANALYSIS USING PRIMERS FL8/FL3 AND N2/N4**

<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR-RFLP</th>
<th>VS2 (FL8/FL3)</th>
<th>VS4 (N2/N4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>E</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>F</td>
<td>F or G</td>
</tr>
<tr>
<td>4</td>
<td>Ha</td>
<td>H</td>
<td>H-K</td>
</tr>
<tr>
<td>5</td>
<td>Hb</td>
<td>L3</td>
<td>H-K</td>
</tr>
<tr>
<td>7</td>
<td>D+H mixed</td>
<td>D+H mixed</td>
<td>D+H-K mixed</td>
</tr>
<tr>
<td>8</td>
<td>F+H mixed</td>
<td>H</td>
<td>H-K</td>
</tr>
</tbody>
</table>

PCR-RFLP = polymerase chain reaction – restriction fragment length polymorphism

important clinically as serovars D, E and F in children. Put together, serovars D, E, F and H caused 97% of all symptomatic and asymptomatic chlamydial infections in the study population groups, including mixed infections, with serovars G and L3 accounting for the rest.

Observed differences in the relative frequencies of serovars between the women in labour and their newborn infants (Table 1) are not unexpected as the samples drawn from the respective population groups were not paired. The two distributions are not, however, significantly different ($\chi^2 = 7.01, 5$df, $p = 0.22$). An investigation of maternal-infant transmission of *C. trachomatis* is being carried out and will be reported separately. On the other hand, the difference observed for *C. trachomatis* serovar F infection between the non-pregnant community-based women in this study and those who presented for delivery at the hospital (Table 1) is noteworthy, implying host preference. Women who were pregnant and older were significantly more likely to be infected with this serovar ($p = 0.003$) than the non-pregnant women drawn from the villages, or women who were pregnant but younger. Results of earlier studies in Japan, however, suggest that there the distributions of serovars E, F and D are similar in these groups of women (27,37). The variable distribution observed for the respective serovars shows that differences in time and place, as well as age and parity, can influence serovar distribution. To resolve these questions of host and disease associations in PNG communities would require a specifically designed study.

We have previously (42) reported that, despite an apparent absence of clinical LGV in PNG communities, the presence of serovar L3 as confirmed in this study shows that the potential for this disease does exist. The epidemiology of LGV is poorly defined. Unlike infections with serovars A-K, which are largely confined to mucosal columnar epithelial surfaces of the genital tract and the eye, the so-called LGV serovars primarily infect macrophages and monocytes. They pass through the epithelial surfaces to access the regional lymph nodes from where they can also cause disseminated infections. However, since this infection cannot be distinguished clinically from other causes of genital ulcerative disease associated with bubo formation, it is quite possible that some LGV may have been misdiagnosed in the past as syphilis, chancroid, herpes or donovanosis in PNG communities. A relatively higher frequency of serovar L3 seen among children presenting with eye discharge (10%) in this study (Table 1), compared to its low genital prevalence and the apparent absence of clinical LGV, suggests that serovar L3 may be causing the same spectrum of pathology associated with non-ulcerative STI serovars D-K in this population. Although this has not been shown to occur
elsewhere, the observed clinical association in this study is consistent with the established similarity of serovar L3 to the other members of the C serocomplex group at the molecular level, namely serovars H, I, J and K (28).

Mixed serovar infections were also detected but found to be uncommon (3%) in the studied population groups. An earlier study (43) showed that mixed serovar infections can be expected in instances where a history of multiple sex partners is present. In the present study, double infections involved the common types. The combinations DE, DF, DH, EF, EH and FH were detected, with mixed serovars DF, DH and EF determined to be more common (4, 3 and 2 individuals respectively). Detection of one unresolved case with multiple atypical DNA bands suggested the possibility of an infection involving more than two serovars. Both this and the presence of new variants have been suggested to occur elsewhere (44), but appear to be rare, as we have also found. We were not able to resolve this particular issue in our study due to failed reamplification attempts.

We did not detect serovars A-C associated with trachoma in this study. This potentially eye-blinding disease has not been rigorously documented in PNG in the past, although it has been looked for clinically (45) and described in population surveys (46); furthermore, D.M. Graham (1982, unpublished data) has reported having detected antibodies to this group of serovars in the ocular samples she obtained. Established genetic and antigenic similarities between serovar Ba and serovars D-K (47) and its association with genital infection implicate it as the most likely causative agent. In view of these observations, a more extensive study would be warranted to confirm the presence or absence of serovar Ba as well as those of serovars A, B and C in PNG.

Infections with these serovars are treatable. Standard treatment with a combination of amoxycillin, probenecid, Augmentin and doxycycline, followed by six days of doxycycline, though broadly efficacious against C. trachomatis as well as N. gonorrhoeae, was not, however, found to be effective in reducing rates of STIs, probably because of the low patient compliance observed in PNG communities. This regimen has now been replaced with a single oral dose of azithromycin (1 g stat), for syndromic treatment of both C. trachomatis and N. gonorrhoeae, which should result in an improved outcome. The apparent lack of broad immunological protection induced by serovars of C. trachomatis and increased potential for infections with alternative types leading to subsequent immunopathology emphasize the need for unwavering adherence to the recommended treatment algorithms.

In all, we have shown in this study that the same major serovars of C. trachomatis responsible for urogenital infections detected in populations elsewhere are present and circulating in communities in EHP. Our results show that beside serovars D, E and F, serovar H can be a common pathogen of the genitourinary tract and can colonize the eyes and nasopharynx of children as well. While a more rigorous study would be required to verify the presence of variants, or host and disease associations with specific serovars, the overall serovar distributions in the population groups of our study generally conform to previously published global patterns.

The PCR-based endonuclease digestion method employed in the study may be sensitive and specific and holds promise for rapid genotyping of C. trachomatis serovars as demonstrated in the overall agreement of its results with the sequencing data and its capacity to distinguish between the detected serovars and possible genovariants. Despite this, our continued failure to amplify some samples, even after they were purified, shows that there is a need to further optimize its application in our setting. On the basis of continued PCR failure under optimized conditions, we now think that this may be due to an error in the design of the reverse primer (primer CT6) used in the study: instead of incorporating an analogue for bases ‘T/C’ in the primer to accommodate reported variability at this site (position 1177) in the C. trachomatis genome (35, 48), only base ‘T’ was incorporated. This is crucial as the exact complementary sequence at the 3'-hydroxyl terminal is critical for primer binding, template extension and a successful outcome during PCR. Correcting this should result in a better outcome in future studies.

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