Original Article

Systemic Antibody Response to *Chlamydia Trachomatis* Infection in Patients Either Infected or Reinfected with Different *Chlamydia* Serovars

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Abstract

Introduction: *Chlamydia trachomatis* is the etiological agent for the most prevalent bacterial sexually transmitted infection in both developed and developing countries. The aim of present study was to characterize the antibody response between two groups of individuals, having either a single *C. trachomatis* infection and or repeated infections. **Material and Methods:** Current study consisted of two groups, one with an initial *Chlamydia* infection and a second with repeated infections. A titre based estimation of specific serum (IgG and IgA) levels using ELISA were performed, which further validated by western blot. *In vitro* neutralizing ability of each patient's serum against both homologous and heterologous strains was also determined. **Results:** Individuals infected with one of the *C. trachomatis* serovars D, E or K exhibited a strong systemic antibody response as characterized by ELISA and western blot. These individuals may have developed at least some level of protection as they only represented single infection. By comparison, individuals infected with serovar D, E or F that exhibited low systemic antibody response often presented repeated *C. trachomatis* infections, suggesting an association with poor immune response. An *in vitro* neutralizing level of 60-90% was observed in the human sera against homologous serovar D and two heterologous *C. trachomatis* serovars E and K, compared to <40% against heterologous serovars F. **Conclusion:** Individuals infected with serovars D and K showed a potential association between circulating antibody response and re-infection risk. While the patients infected with serovars E showed a disconnection between systemic antibody response and re-infection risk.

Keywords: *Chlamydia trachomatis,* IgG, *in vitro* neutralisation, serological response

Introduction

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted infection (STI) in humans.[1] The prevalence of *C. trachomatis* infection has been rising progressively in many countries, with >100 million new cases estimated annually around the globe (WHO).[2] An estimated 3–4 million new cases occur every year in the US, 5 million in Western Europe and 16 million in Sub-Saharan Africa.[3] The largest burden of *C. trachomatis* infection occurs in women, where complications can include pelvic inflammatory disease. However, because patients with *C. trachomatis* urogenital infections often do not exhibit any symptoms (75%–90% of patients), they remain undiagnosed and untreated. This can lead to tubal factor infertility, miscarriage or ectopic pregnancy.[4-6] Genital *Chlamydia* infections also increase the susceptibility to other sexually transmitted agents, such as HIV.[7]

Repeated chlamydial genital infections are common and account for a substantial proportion of incident infections.[8] Although *C. trachomatis* infections can be treated effectively with antibiotics such as azithromycin, or doxycycline, almost one-fourth of individuals are re-infected with *C. trachomatis*.[9] Repeated infections result from failure of antibiotic therapy or from reinfection due to continued unprotected sexual contact

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with either an untreated existing partner or a new infected partner.[8] *Chlamydia* reinfection incidence and treatment failure is rising with high repeat *C. trachomatis* infection rates observed in community cohorts of women in the UK (25.5%) and among women attending general practice clinics in Australia (22.3%) and the UK (29.9%).[10]

In the current study, we identified two groups of women (single infection versus repeat infections) and studied their serum antibody response. We aimed to gain insight into why some of the individual women get reinfected with *Chlamydia* while some of them only have a single infection. The specific aim of the present study was to characterise the systemic antibody responses (IgG and IgA) of these two groups of individuals against semi-purified elementary bodies (EBs) of *C. trachomatis* serovar D.

Materials and Methods

Chlamydia trachomatis cell culture and preparation of semi-purified elementary bodies

C. trachomatis serovar D (ATCC® VR-885™) EBs were prepared by infecting HEp-2 cell lines (ATCC® CCL-23) in the presence of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Australia) containing 5% heat-inactivated foetal calf serum (FCS) (Life Technologies, Australia), 120 μg/ml streptomycin (Sigma-Aldrich, Australia) and 50 μg/ml gentamycin (Gibco, Australia), 37°C, 5% CO_2.

Once cells reach confluence (>90%), they were infected with *C. trachomatis* serovar D EBs in sucrose phosphate buffer (SPG) by centrifugation at 500 ×*g* at 28°C for 30 min, and afterwards incubated in DMEM containing 2% FCS and cycloheximide (1 μg/ml) to inhibit HEp-2 cell protein synthesis. 48–50 h postinfection, media was removed from the infected flask and replaced with ice-cold SPG. The *C. trachomatis*-infected monolayer was scraped from the flask and added to the fresh falcon tube containing glass beads (Sigma-Aldrich, Australia) and further vortexed it. The suspension was then centrifuged at 500 ×*g* for 10 min at 4°C and the supernatant centrifuged at 18,000 ×*g* for 30 min at 4°C. The semi-purified EB was resuspended in SPG and stored at −80°C.[11-13]

Serial dilutions of the suspension obtained were used for EB quantification. HEp-2 cells were fixed and stained with monoclonal antibody-fluorescein isothiocyanate. The mean number of inclusion-forming units (IFUs) of the single dilutions, counted at the epifluorescence microscope (Leica DMLB), was used to calculate the suspension titre expressed in IFU/ml.[14-16]

Chlamydia trachomatis serology

Enzyme-linked immunosorbent assay

Serum IgG and IgA antibody titres for each individual were determined, as described by Carey *et al*., 2010,[17] at two different time points (for individual with both single infection, as indicated with suffix 3, as well as repeated infection, as indicated with suffix 15) as well as at single time point (for individuals having single infection only as indicated with suffix 3). Two-fold dilutions of serum (50 μl/well) were added to the wells of flat-bottom 96-well plates (ThermoLab systems) coated with semi-purified EBs of *C. trachomatis* D (5×10^4 EBs per well).

Western blot

Western blot assay was used to assess the expression of IgG and IgA antibodies of an individual serum sample specific to *C. trachomatis* D semi-purified EBs (1.75×10^5) and was performed as described previously.[18] In brief, ~30 μg of purified EBs was loaded on 0.75 mm-wide 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels (110 V for 1 h). Following transfer to a nitrocellulose membrane (Pall Corporation, Australia) at 90 V for 1 h, membranes were blocked for non-specific binding in blocking buffer (5% skimmed milk in 1× Tris-buffered saline) overnight at 4°C or for 2 h at room temperature. For the post-blocking, serum samples were added on to the membrane at a dilution of 1:1000 in blocking buffer and incubated overnight at 4°C or 2 h at room temperature. Membranes were then washed 4 times with 1 × TBS-T for 5 min each. Secondary antibody anti-human IgG and IgA in goat were added at 1:1000 dilutions in blocking buffer and incubated for 1 h at room temperature. Membranes were again washed 4 × with TBS-T for 5 min each. Finally, tertiary antibody (Donkey anti-goat IgG horseradish peroxidase [HRP] conjugate [for IgG detection] and Rabbit anti-goat IgA HRP conjugate [for IgA detection] Southern Biotech/Invitro Technologies, Cleveland, Australia) was separately added onto the membranes at 1:1000 in blocking buffer for 1 h at room temperature. Membranes were then washed 5 × with TBS-T for 5 min each. Blots were visualised for bands by adding enzymatic chemiluminescence substrate (Thermo Fisher Scientific, Australia).

In vitro chlamydia neutralisation assay

In vitro neutralisation assay was performed using individual's serum samples collected at two different time points (individual with single and repeated infection) as well as single time point, according to Kollipara *et al*.[18] Both cells and inclusions were counted under the microscope, and a mean of ten fields of view for each well was counted and the neutralisation percentage was determined and compared to media-only controls.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, LaJolla, CA, USA). Data were presented as mean ± standard deviation from triplicate assays. For statistical significance, data were analysed using one-way ANOVA and Kruskal–Wallis (nonparametric) tests. The *P* value for significance was set at ≤0.05.

Results

In the present study, the serum samples were obtained from a cohort (n = 22) of women (enrolled in the Australian *Chlamydia* Treatment Study, ACTS), infected with different

Chlamydia serovars. Out of these 22 individuals, five women were infected with serovar D, one was infected with serovar K, 12 were infected with serovar E and four were infected with serovar F. The systemic antibody response (IgG and IgA) was measured via enzyme-linked immunosorbent assay (further validated through Western blot) among the individuals (i.e., homologous vs. homologous and homologous vs. heterologous) having single as well as those having repeated infections. Further, the neutralising ability of different serum samples (infected with different *Chlamydia* serovar) was analysed against semi-purified EBs of *C. trachomatis* serovar D.

Systemic antibody responses of patients infected with *Chlamydia trachomatis* serovar D or K showed correlations with reinfection risk

The results showed that some of the individuals (40%) infected with serovar D exhibited high IgG and IgA antibody titres [1-020-3, 1-186-3, Figure 1a] as well as exhibiting high neutralisation level in the *in vitro* assay [Figure 1c]. Hence, we propose that they may have had some level of protection against reinfection; patient 1-193-3 was an exception [Figure 1]. Some individuals (1-082-3 and 1-233-3) also exhibited a high IgG titre but exhibited low neutralisation ability, and we propose that they were not protected against subsequent *C. trachomatis* infections. Similarly, in Western blot, there was a corresponding increase in IgG response observed among the individuals with high IgG titres [Figure 1b]. The individual infected with serovar K (only one patient was infected with serovar K, 1-049-3) also exhibited a high systemic antibody response, as well as high neutralising ability [60%; Figure 2], towards infections, and was not reinfected.

Systemic antibody responses of patients infected with *Chlamydia trachomatis* serovar E showed a disconnection between immune response and reinfection risk

The level of IgG and IgA antibodies as well as the *in vitro* neutralising ability was also analysed among the individuals ($n = 12$) infected with serovar E (analysed against serovar D). The results showed that one of the individuals (infected with serovar E, 1-139-3) exhibited a high IgG titre (IgG) as well as a high neutralising (60%) ability and was not reinfected [Figure 3]. Individuals 1-244-3 and 1-235-3, despite exhibiting a high IgG titre and high neutralising levels (63-70%), were still reinfected with *Chlamydia*. Individuals 1-103-3, 1-052-3, 1-202-3 and 1-010-3, exhibited a very high IgG titre but with low neutralising ability (5%–40%) towards infection and were found to become reinfected within 6-month study period [Figure 3].

Systemic antibody responses of patients infected with *Chlamydia trachomatis* serovar F showed correlations with reinfection risk

The level of IgG and IgA antibodies as well as the *in vitro* neutralising ability was also analysed among the individuals ($n = 4$) infected with serovar F (analysed against serovar D). The results showed that individuals 1-124-3, 1-166-3 and 1-156-3 exhibited neither high IgG titre nor high neutralising (5%–36%) levels, and therefore, perhaps not surprisingly were reinfected, except 2-595-3 [Figure 4].

The systemic antibody response of patients re-infected with *Chlamydia trachomatis*

Finally, we analysed the systemic antibody response among the individuals, who had repeated *C. trachomatis*

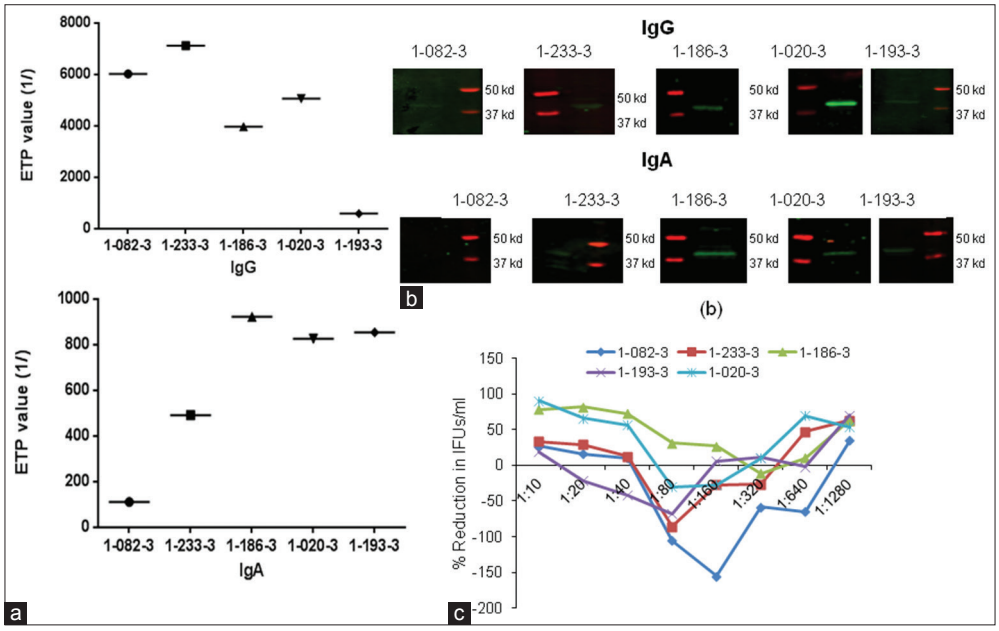

Figure 1: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after initial *Chlamydia trachomatis* infections infected with serovar D and tested against semi-purified elementary bodies of serovar D

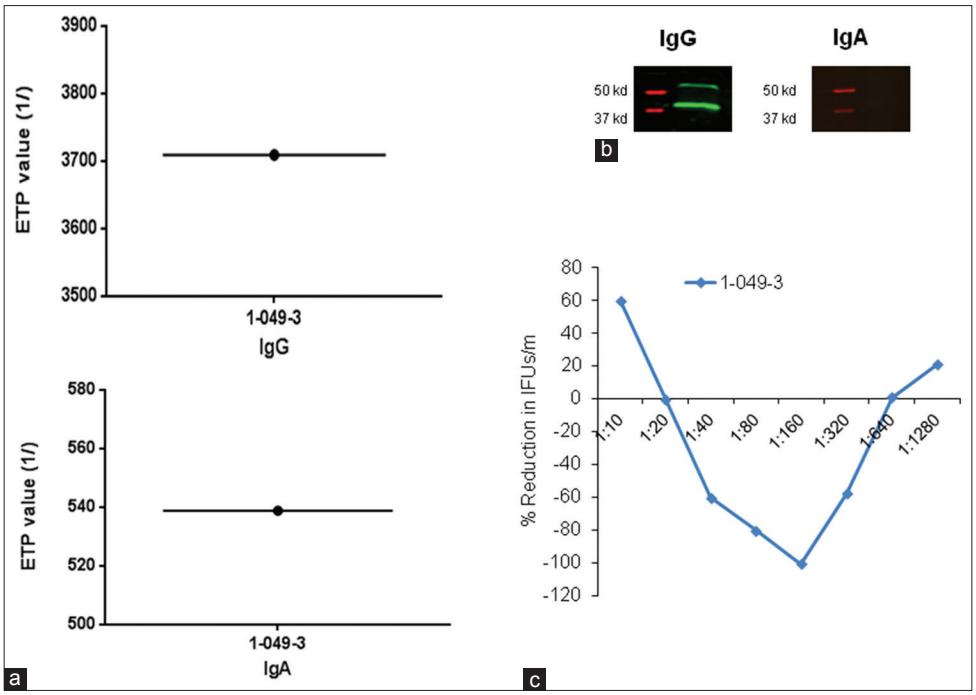

Figure 2: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after initial *Chlamydia trachomatis* infections infected with serovar K and tested against semi-purified elementary bodies of serovar D

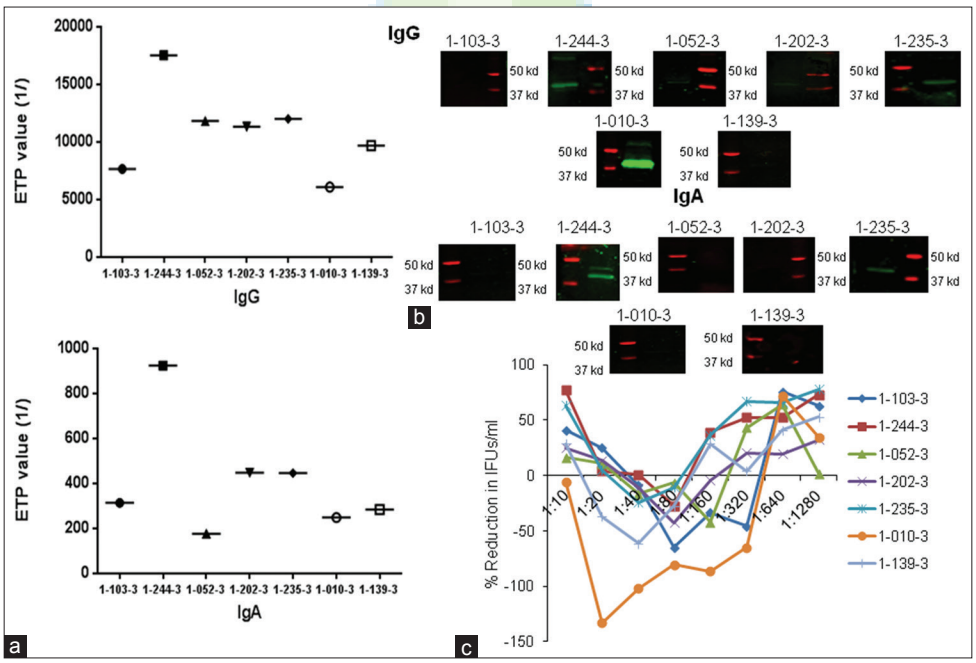

Figure 3: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after initial *Chlamydia trachomatis* infections infected with serovar E and tested against semi-purified elementary bodies of serovar D

infections. A high IgG and IgA response, as well as high neutralisation level (75%–90%), was observed among the individuals (1-082-15, 1-233-15) infected with serovar D, when tested against the same serovar D [Figure 5]. The same trend was also observed among the individuals infected with different serovars and analysed against serovar D. Similarly, high IgG and IgA responses as well as high neutralisation levels (52%–97%) were also observed among the individuals (1-103-15, 1-244-15, 1-052-15, 1-235-15, 1-010-15, 1-610-15, 1-212-15, 1-131-15 and 1-586-15) infected with serovar E, when tested against the same serovar D [Figures 6 and 7] except 1-202-15. Although a high

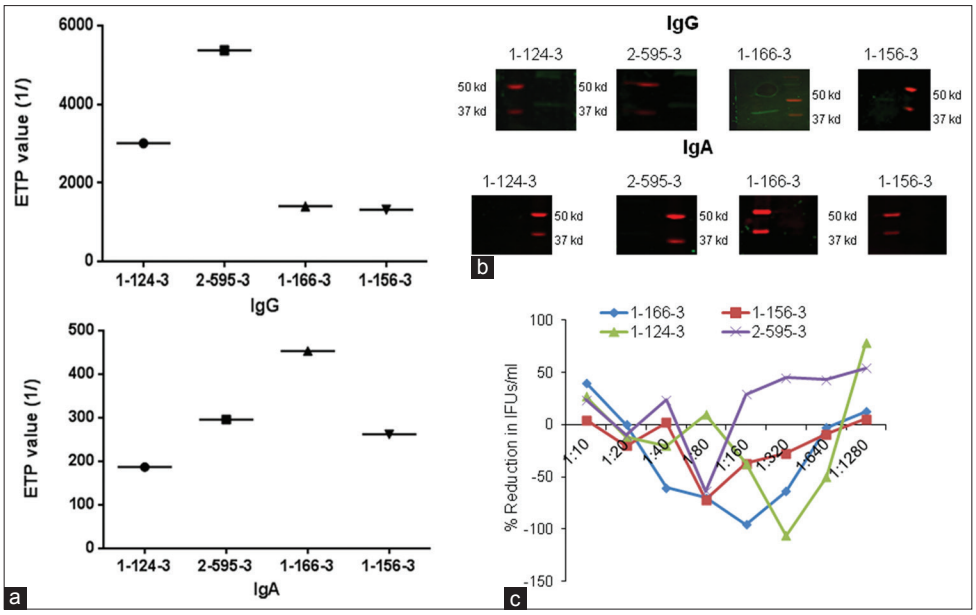

Figure 4: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after initial *Chlamydia trachomatis* infections infected with serovar F and tested against semi-purified elementary bodies of serovar D

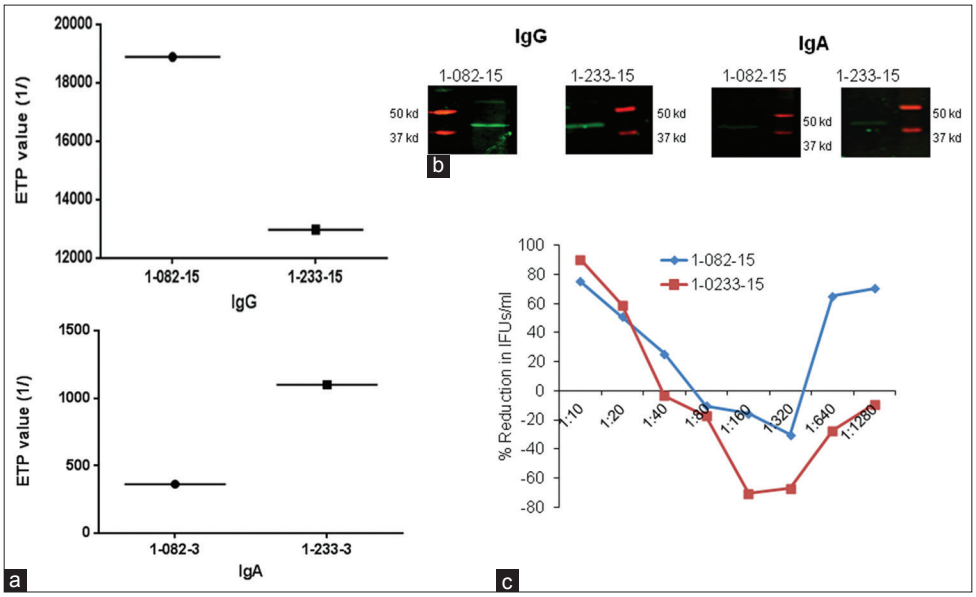

Figure 5: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after repeated *Chlamydia trachomatis* infections infected with serovar D and tested against semi-purified elementary bodies of serovar D

IgG response was also observed among individuals infected with serovar F (1-124-15, 2-595-15) and analysed against D, these patients exhibited low neutralisation levels (<40%) towards infection [Figure 8].

Discussion

C. trachomatis is the aetiological agent for the most prevalent bacterial STI in both developed and developing countries. The diagnostic rates for *C. trachomatis* infection have increased dramatically over the last decade.[19,20] Repeated infections of *C. trachomatis* are very common and may represent reinfection from an untreated partner or treatment failure.[21]

The individuals included in this relatively small study were studied over a 6-month period and any infection/reinfection cases were included and recorded. The individuals were initially treated with a single oral dose of 1 g azithromycin at the time of recruitment into the ACTS trial,[17] subsequent to their diagnosis of their initial *Chlamydia* infection (infected with different *Chlamydia*

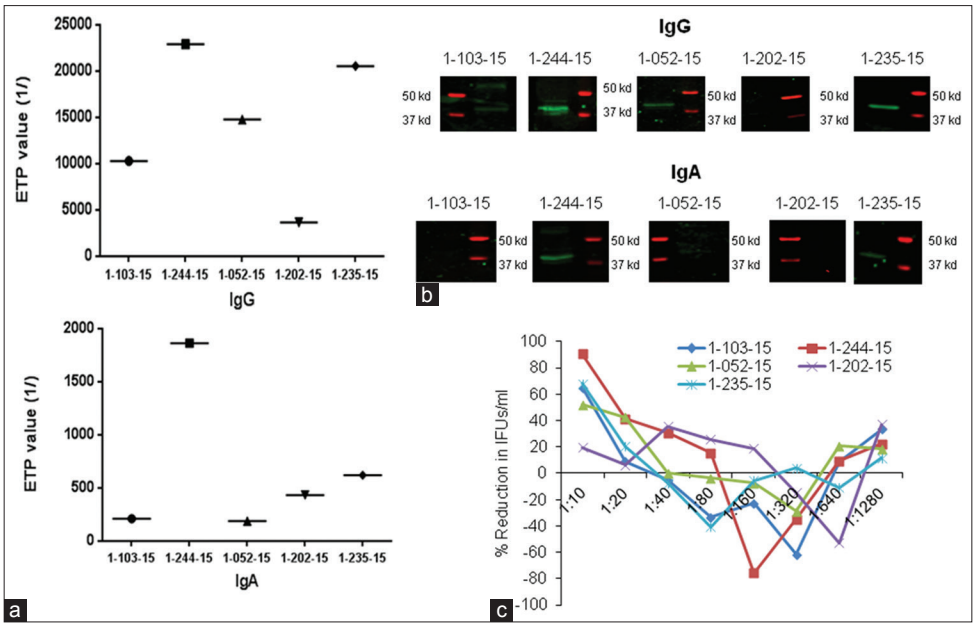

Figure 6: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after repeated *Chlamydia trachomatis* infections infected with serovar E and tested against semi-purified elementary bodies of serovar D

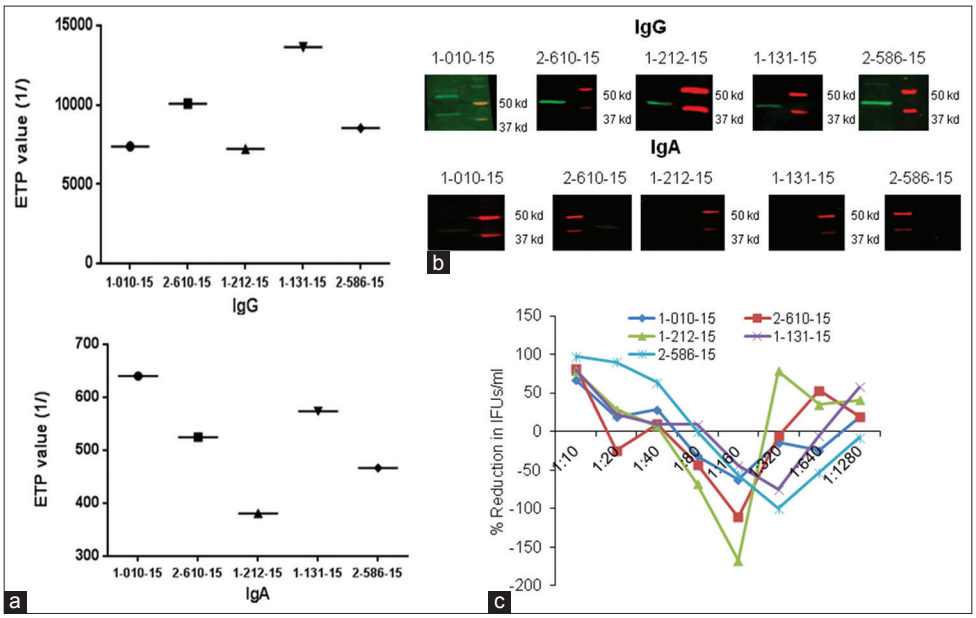

Figure 7: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after repeated *Chlamydia trachomatis* infections infected with serovar E and tested against semi-purified elementary bodies of serovar D

serovars). It has previously been reported that neutralising species-specific or serovar-specific antibodies can be produced in response to *C. trachomatis* infection in humans, as well as in some animal species.[21] Our results showed that a strong humoral immune response, as characterised by high serum antibody titres combined with high levels of *in vitro* neutralising antibodies (>60%) in serovars D and K, may have been associated with some level of protection against reinfections in these individuals. By comparison, some other individuals, also infected with serovar D, had low neutralisation capacity (<60%), and subsequently these individuals were reinfected, indicating no significant level of protection. This is in contrast to what was seen in some of the patients infected with serovar E, where individuals with a high neutralisation (40%–75%) capacity were still reinfected. This suggests subtle differences in epitopes between serovars D and E and this may relate to their *in vivo* protective ability.

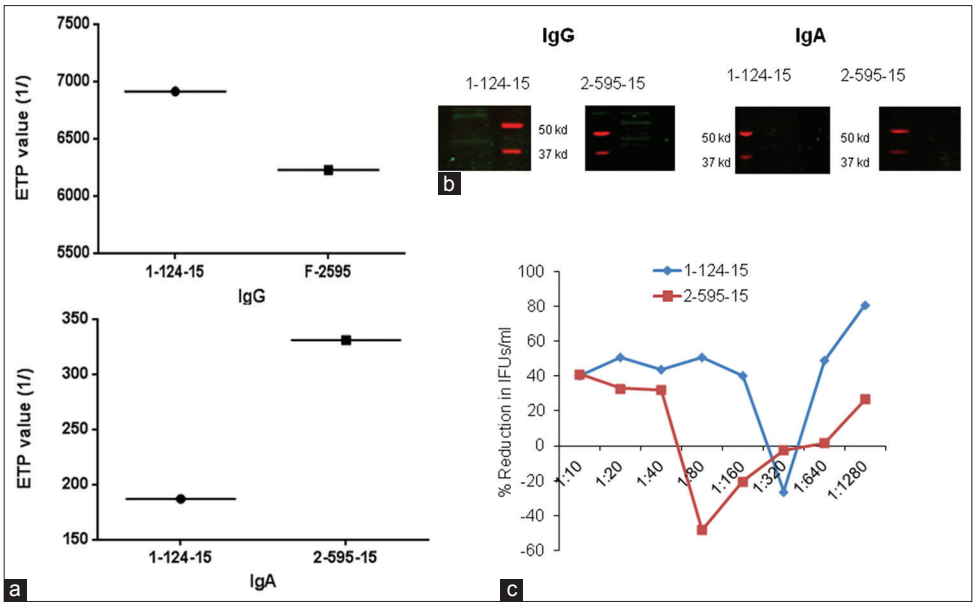

Figure 8: Systemic antibody response, IgG and IgA, as measured by (a) enzyme-linked immunosorbent assay, (b) Western blot and (c) *in vitro* neutralisation level of serum sample among individuals after repeated *Chlamydia trachomatis* infections infected with serovar F and tested against semi-purified elementary bodies of serovar D

Conclusion

This study shows that individuals infected with serovars D or K exhibited a high systemic antibody response (IgG and IgA) as well as high neutralisation levels against serovar D except those individuals who were infected with serovar F. Individuals infected with serovars D and K showed an association with humoral immune response and reinfection risk (i.e., high immune response = low reinfection risk). Similarly, individuals infected with serovar F also showed an association with immune response and reinfection risk (i.e. low immune response = low reinfection risk). Although some of the individuals infected with serovar E exhibited a high systemic antibody response as well as high neutralisation level against tested serovar D, they showed disconnection between associations with humoral immune response and reinfection risk (i.e., a high immune response did not always result in protection from reinfection).

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Conflicts of interest

There are no conflicts of interest.

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