Original Article

Seroprevalence of antibodies against *Chlamydia trachomatis* inclusion membrane proteins B and C in infected symptomatic women

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Abstract

Background: Proteins in the inclusion membrane of *Chlamydia trachomatis* (CT) have been anticipated to play pivotal roles in the molecular and cellular interactions between the pathogen and host. However, there is lack of data on host immunity with respect to antibody responses against chlamydial inclusion proteins.

Methodology: We used full-length fusion proteins for CT inclusion membrane proteins B and C (IncB and IncC respectively), two early-infection phase proteins, to study their role in antibody generation during human infection.

Results: Three hundred and fifty-five women (aged 22-36 years) attending the Gynaecology outpatient department, Safdarjang Hospital, New Delhi, India were enrolled in this hospital ethical committee approved study. Out of these, 108 were diagnosed to be cervical CT-positive. Of these 108 patients, 67 (62.03%) showed ELISA positivity for IncB IgG, and 64 (59.25%) for IncC IgG. There was a positive correlation between antibody titres against IncB and IncC and with antibodies against CT major outer membrane protein (MOMP) in CT-positive sera. Our data also showed a positive association between antibody titres against IncB and IncC in patients with cervicitis and pelvic inflammatory disease (PID). Significantly high antibody titres were detected in cervicitis cases compared with PID. There were significantly higher levels of serum cytokines (TNF-α, IFN-γ and IL-12) in Inc-positive cervicitis cases than in PID cases. In addition, our study also showed higher IncB and IncC IgG_2 titres in comparison to respective IgG_1, IgG_3 and IgG_4 titres in CT-positive sera.

Conclusion: Our data suggested that antibodies against CT IncB and IncC were prevalent in CT-positive women diagnosed with cervicitis or PID.

Key words: *Chlamydia trachomatis*, inclusion membrane proteins, seroprevalence, cervicitis, infertility

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Introduction

Chlamydia trachomatis (CT) is the most common bacterial sexually transmitted infection worldwide, especially among young adults [1]. Chlamydial infections are asymptomatic in the majority of patients and hence often remain undiagnosed. Undiagnosed and untreated chlamydial infections can ascend to the upper genital tract, where they colonize the endometrial mucosa and the fallopian tubes, leading to pelvic inflammatory disease (PID). Early detection is hence judicious for preventing established infection within the human host.

Although the serovars of CT have differential host tissue tropisms [2,3], they are very similar genetically [4,5], and share a common intracellular biphasic growth cycle [6] within a non acidified cytoplasmic vacuole termed as an inclusion [7]. The inclusion membrane is very critical in its capacity as an interface between this intravacuolar pathogen and the infected host cells. The inclusion membrane facilitates the exchange of nutrients and metabolites between host and chlamydiae [8-10], secretion of chlamydial factors to the infected host cells [11], and modulation of the latter's signalling network [12].

The several chlamydial inclusion proteins termed "Incs" [13] localized to the inclusion membrane have the potential to play key roles in this host-pathogen interaction and thus have become an important area of research. Expression of chlamydial Incs early in the infectious process suggests that their involvement in inclusion modification is crucial to the outcome of host-chlamydiae interactions [7]. Incs are considered to be mediators of host-chlamydiae interactions as their hydrophilic domains localized on the outer surface of the inclusion membrane are phosphorylated by host kinases [14,15]. Chlamydial incs are secreted through the type III secretion apparatus [16] and incorporated into the host phagosomal membrane [17]. Although these proteins may provide contact with the host cell, their role in

the development of host immunity against infection is yet to be clearly elucidated.

With the recent availability of literature on comparative immunogenicity of chlamydial inclusion proteins [18], we assessed the role of two Incs of CT, namely inclusion proteins B and C (IncB and IncC respectively) in generating humoral immune responses in CT-infected women diagnosed with cervicitis or PID/ infertility. These two proteins, with homologues in *C. pneumoniae* [19], *C. psittaci* [20], *C. muridarum* [4], and *C. abortus* [21], belong to the early phase of infection where their gene expression begins within a half hour post infection of the host cell and is simultaneous with inclusion formation and its transportation into the perinuclear space, and evasion of fusing with early lysosomes [22]. These features suggest that IncB and IncC might play a significant role at early stages of chlamydial infection development and provide necessary elements in processes of inclusion formation.

This study thus aimed to evaluate the seroprevalence of *C. trachomatis* infection by measuring antibody levels against IncB and IncC with respect to anti-MOMP antibodies in sera obtained from symptomatic women. We correlated the titres of antibodies against IncB and IncC with severity of disease in these CT-positive women diagnosed with cervicitis or PID/infertility.

Materials and Methods

Study population

A total of 355 women (aged 22 to 36 years) attending the outpatient department of Safdarjang Hospital, New Delhi, India, for gynaecological complaints (cervical discharge, lower abdominal pain, pelvic pain, ectopy, erosion, PID and infertility) were enrolled in the study. They were confirmed as symptomatic after careful physical examination. Of these, 163 patients were diagnosed with cervicitis (presented with mucopus in endocervical exudates) while 76 had PID/infertility. Findings at diagnostic laparoscopy/ hysterosalpingogram, viz. tubal patency, adhesions, hydrosalpinx formation and endometriosis were noted for infertile patients. Infertile women were identified as those who lack recognized conception after 1.5 to two years of regular intercourse without the use of contraception. The study was approved by the hospital ethical committee and informed written consent was obtained from each patient. On recruitment, a detailed history was taken from each patient.

Collection of samples

The vulva and cervix were examined for evidence of lesions and vaginal/cervical discharge. After cleaning the endocervix with a cotton swab, two cervical specimens were collected on separate cotton swabs and placed in sterile vials containing phosphate-buffered saline (PBS) [22,23]. Cells were extracted by vortex mixing, and then direct fluorescent assay (DFA) and polymerase chain reaction (PCR) methods were used to detect endocervical CT infection [24-26].

Diagnosis of other sexually transmitted disease (STD) pathogens was done by culture (*Neisseria gonorrhoeae, Mycoplasma hominis, Ureaplasma urealyticum*) and microscopy of Gram-stained smears (Candida spp., bacterial vaginosis, and *Trichomonas vaginalis*). Non-heparinised venous blood was drawn; the serum was separated and then stored at –70°C for the detection of antibodies against MOMP, IncB and IncC and for measuring serum cytokines.

Cloning and Expression of CT MOMP, IncB and IncC proteins

Full-length gene sequences of MOMP, *incB* (CT 232) and *incC* (CT 233) available from the CT serovar D genome (http://www.berkeley.edu:4231; http://www.stdgen.lanl.gov/) were cloned into pGEX vectors (Amersham Pharmacia Biotech, Inc., NJ, USA) and expressed as fusion proteins with glutathione S-transferase (GST) fused to the N terminus of the chlamydial proteins. Production of GST fusion proteins was performed as described previously [18]. Briefly, production of GST fusion proteins were induced with isopropyl-D-thiogalactopyranoside (IPTG; Invitrogen, CA, USA) and GST fusion proteins were extracted by lysing the bacteria via sonication in a Triton X-100 lysis buffer (1% Triton X-100,1mM phenyl methyl sulfonyl fluoride, 75 U of aprotinin/ml, 20M leupeptin, and 1.6M pepstatin). After a high-speed centrifugation to remove debris, the fusion protein-containing supernatants were further purified with glutathione-conjugated agarose beads (Amersham Pharmacia Biotech, Inc., NJ, USA) for use in further assays.

Detection of antibodies against CT MOMP, IncB and IncC

The MOMP, IncB and IncC specific antibody titres in sera were determined by specific Enzyme-linked immunosorbent assay (ELISA) as previously described [27]. Briefly, 96-well plates were coated with 1 µg antigen/well and 100 µl patient sera

samples were added per well in serial dilutions. Free GST was coated in separate wells to serve as negative controls. After incubation for two hours at 37°C and subsequent washing with PBS-Tween 20 (PBS-T), plates were incubated with horse radish peroxidase (HRP) - conjugated rabbit anti- human IgG (1:10,000 dilutions), IgG_1, IgG_2, IgG_3 and IgG_4 (all 1:2000 dilutions) antibodies (Bangalore Genei, Bangalore, India). The binding was measured in an ELISA reader using OPD (o-phenylenediamine dihydrochloride) as the substrate. Positive samples were defined as those yielding an absorbance (OD) value at least two standard deviations (SDs) above the mean value obtained from the panel of samples taken from the negative subjects.

Quantification of serum cytokines

Quantification of interleukin-1ß (IL-1ß), IL-4, IL-10, IL-12, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) was done using ELISA kits (eBiosciences, San Diego, CA, USA), in accordance with the manufacturer's instructions. A log-log standard curve was generated, and unknowns were interpolated. The sensitivities of cytokine kits were 2 pg/mL. Results from test samples were compared with control sera obtained from 25 healthy women attending the family planning department for regular checkups who were also enrolled in the study.

Statistical analysis

Statistical Analysis was performed with Graphpad Prism Version 5 (La Jolla, CA, USA). Spearman's rank method was used to find any correlation between anti-chlamydial antigens. The level of significance among groups was compared using the $\chi 2$ test. Significance of antibody titres was calculated by independent t-test.

Results

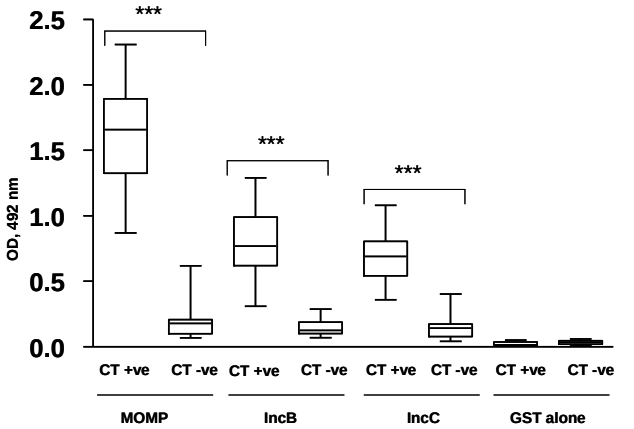
Diagnosis of STD pathogens in the cervix

Cervical CT infection was diagnosed in 108 (30.2%) patients. These patients were found to be uninfected with other STD pathogens. Among the CT-negative patients, 13 (5.1%) were infected with Candida spp., 28 (11.3%) had bacterial vaginosis, 24 (9.4%) were infected with *M. hominis*, and 61 (24.7%) with *U. urealyticum*. None of the patients had *N. gonorrhoeae or T. vaginalis*.

Detection of antibodies against MOMP, IncB and IncC

ELISA results for anti-MOMP, anti-IncB and anti-IncC IgG antibodies in patients' sera showed significantly higher ($p < 0.001$) OD values in CT-positive patients than in CT-negative patients (median values: 1.660 v/s 0.130, 0.770 v/s 0.067 and 0.690 v/s 0.110, respectively; Figure 1). All samples positive for anti-MOMP, anti-IncB and anti-IncC IgG antibodies had OD values greater than the mean +2SD of that of negative samples. Out of the 108 CT-positive patients, 73 (67.59%) showed ELISA positivity for anti-MOMP IgG, 67 (62.03%) for anti-IncB IgG, and 64 (59.25%) for anti-IncC IgG. Wells coated with GST alone served as negative controls and showed very low antibody titres in CT-positive and CT-negative patients (Mean ± standard deviation; 0.039 ± 0.0012; 0.043 ± 0.0019 respectively).

Figure 1. Quantitation of chlamydial protein-specific antibodies in patients' sera.



Quantitation of chlamydial protein-specific antibodies in human sera by ELISA using recombinant chlamydial proteins is shown. Median values of antibodies against MOMP, IncB and IncC are measured in sera from *Chlamydia trachomatis* positive (CT +ve) and CT negative (CT –ve) sera obtained from women. Wells coated with GST alone served as negative controls. *** represents $p < 0.0001$, *i.e.* highly significant. NS, Not Significant. Y axis = Optical Density (OD) of anti chlamydial antibodies measured at 492 nm; X axis = chlamydial antigens in CT +ve and CT-ve sera.

Anti-MOMP, anti-IncB and anti-IncC antibody titres

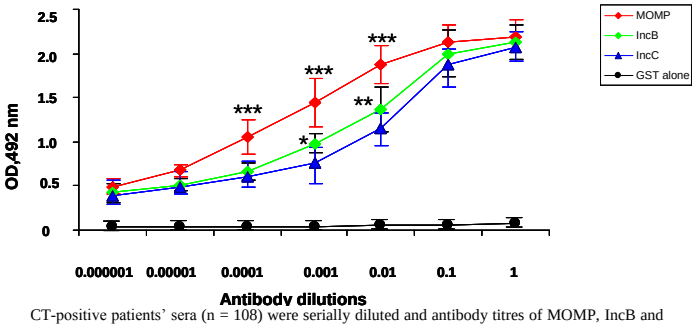
CT-positive sera were serially diluted (1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and 1:1000, 000) and assayed for antibody titres for anti-MOMP, anti-IncB and anti-IncC. Anti-MOMP antibody titre was found to significantly higher ($p < 0.001$) compared to that of anti-IncB and anti-IncC at 1:100, 1:1000 and 1:10,000 dilutions. The anti-IncB titre was found to be higher than anti-IncC at 1:100 ($p = 0.0028$) and at 1:1000 ($p = 0.0189$) (Figure 2).

Correlation between anti-MOMP, anti-IncB and anti-IncC antibody titres

A highly significant positive correlation ($r = 0.3275$, $p = 0.0068$) was seen between anti-MOMP

and anti-IncB antibodies in CT-positive sera (Figure

Figure 2. Antibody titres against CT proteins in serially diluted CT-positive patients' sera.



CT-positive patients' sera (n = 108) were serially diluted and antibody titres of MOMP, IncB and IncC were measured and expressed as mean (OD values) ± standard deviation. Statistically significant differences in OD values between anti-MOMP and anti-IncB were detected at 1:100, 1:1000 and 1:10,000 (P < 0.0001 ***). Anti-IncB antibody titres were significantly higher than that of anti-IncC at 1:100 (P= 0.0028 **) and 1:1000 (P = 0.0189 *) Y axis = Optical Density (OD) of anti chlamydial antibodies measured at 492 nm; X axis = Serial dilutions of sera obtained from CT +ve patients.

3a). Similarly, anti-MOMP and anti-IncC antibody titres were positively correlated (r = 0.3608, p = 0.0034) (Figure 3b). The anti-IncB and anti-IncC antibody titres were also positively correlated (r = 0.2827, p = 0.0236) in these sera (Figure 3c). There was a negative and insignificant correlation between antibody titres against MOMP, IncB and IncC in CT-negative sera.

Anti-IncB and anti-IncC antibody titres in cervicitis and PID/ infertility sera

In CT-positive, IncB ELISA-positive patients (n = 67), 21 (31.34%) were diagnosed with PID/infertility while 38 (56.71%) had cervicitis. In the IncC ELISA-positive patients (n = 64), 17 (25.37%) had PID/ infertility and 45 (67.16%) had cervicitis. Sera from these Inc-positive women were serially diluted (1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and 1:1,000,000) for comparing antibody titres between the cervicitis and PID/infertility groups. Anti-IncB antibody titres were significantly higher in sera of cervicitis patients with respect to those with PID/infertility at dilutions 1:10,000 (p = 0.0017), 1:1000 (p = 0.0154) and 1:100 (p = 0.0002) (Figure 4a). Similar trends were seen for anti-IncC antibody titres; however, titres were significantly higher at sera dilutions 1:1000 (p = 0.0179) and 1:100 (p =0 .0178) (Figure 4b). Anti-MOMP antibody titres were also significantly high in sera of cervicitis patients with respect to those with PID/infertility at dilutions 1:10,000 (p = 0.0014), 1:1000 (p = 0.0135) and 1:100 (p = 0.0034) (Data not shown)

Antibody subtype titres against IncB and IncC in CT-positive and CT-negative sera

In CT-positive sera, anti-IncB IgG_2 and anti-IncB IgG_3 levels were significantly higher ($p < 0.001$) than that in CT-negative sera. Also anti-IncB IgG_2 produced significantly higher titres ($p < 0.001$) than anti-IncB IgG_3 levels in CT-positive sera. Similar trends were seen for anti-IncC IgG_2 and anti-IncC IgG_3 (Figure 5). Low antibody titres (0.01-0.046) were detected in negative control wells coated with free GST. (Data not shown)

Cytokines concentrations in Inc-positive cervicitis and PID/ infertility sera

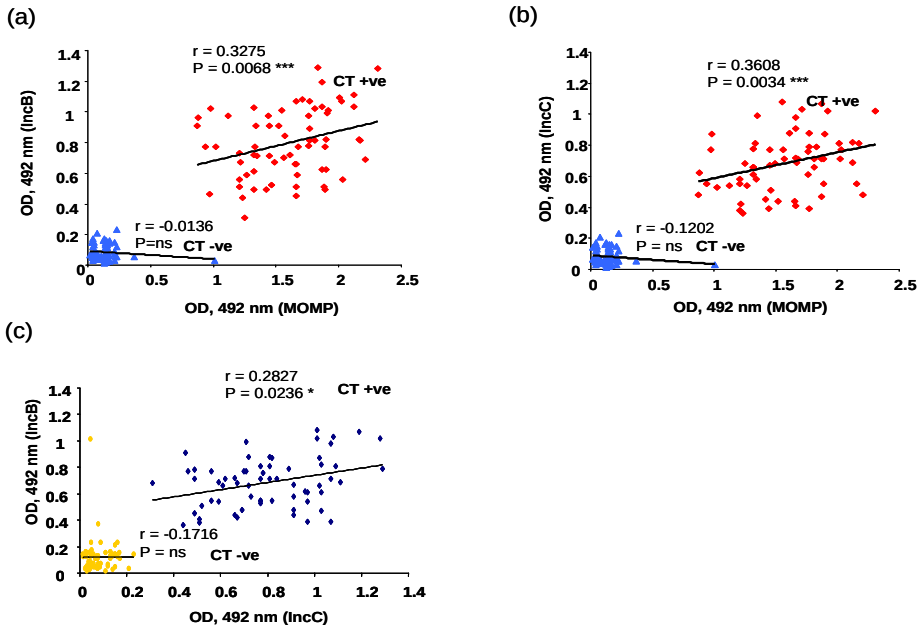
Median concentrations of cytokines in serum samples of MOMP-positive, Inc-positive women with cervicitis or PID/infertility are given in Table 1. Median IFN-γ, IL-12 and TNF-α levels were higher in CT IncB or CT IncC positive cervicitis women with women diagnosed with PID/Infertility. Serum IL-4 levels were under the detection limit in all serum samples. No significant difference was observed between levels of IL-1ß in IncC-positive patients. Cytokine levels in MOMP-positive, Inc-positive women were higher than those in CT-negative patients or healthy controls (data not shown).

Discussion

Chlamydiae actively modify the vesicular interactions of the inclusion very early in the infectious process to create a protected intracellular niche. Many of these interactions are controlled by chlamydial proteins located at the cytoplasmic face of the inclusion membrane [28]. Several Inc proteins have been identified in CT and there is recent literature on the characterization and location of Incs [18] but their role in host pathogen immunity is not well elucidated. Further, there is lack of information on the probable association of CT Incs with disease pathologies in patients with genital chlamydial infection.

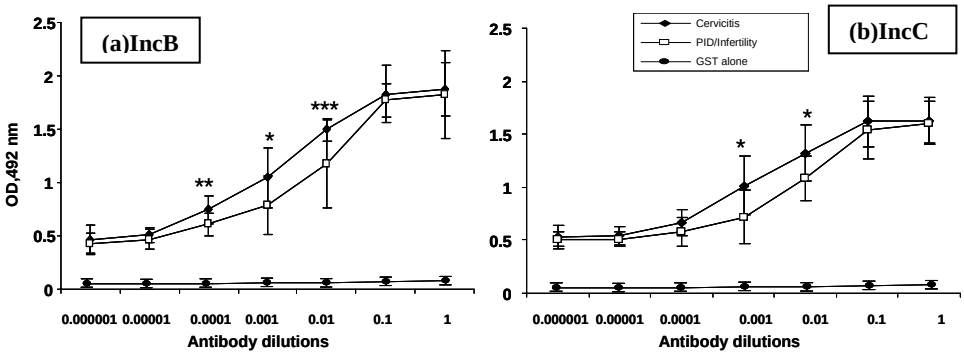
The results of this study agreed with previous data which showed 23-30% incidence of chlamydial infection in the lower genital tract [22,25,29,30]. Using an anti-recombinant protein antibody approach, we were able to detect antibodies against IncB and IncC in 62.03% and 59.25% respectively in CT-positive women. Furthermore, antibody titres

Figure 3. Correlation of antibody titres against MOMP and Incs in CT-positive and CT-negative patients' sera.



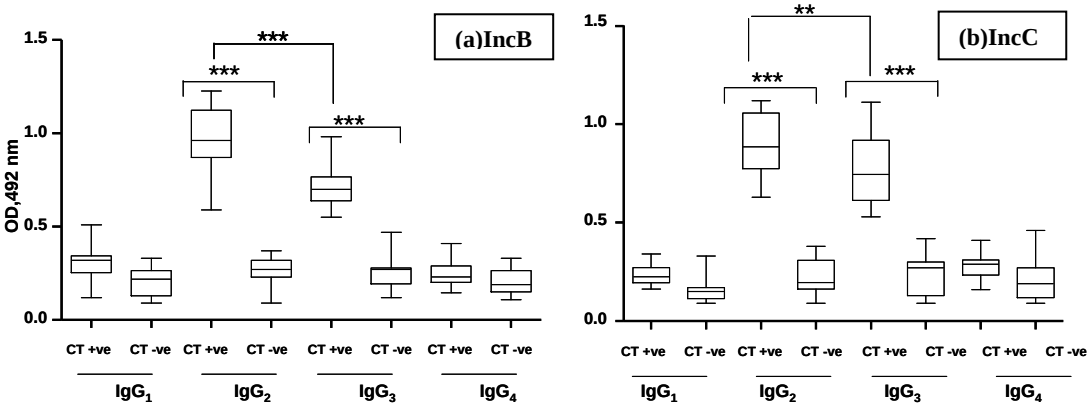
Scatter plot showing the correlation of the serological response to immunogenic chlamydial proteins: (a) anti-MOMP versus anti-IncB; (b) anti-MOMP versus anti-IncC and (c) anti-IncB and anti-IncC. There is positive correlation (r) between respective antibody titres in CT-positive sera and negative, insignificant correlation in CT-negative sera.

Figure 4. Antibody titres against CT Incs in serially diluted sera of CT-positive patients diagnosed with cervicitis or PID/infertility.



CT-positive sera (n = 108) were serially diluted and antibody titres of IncB and IncC were compared between cervicitis and PID/infertility cases: a) Comparison of OD values of anti-IncB showed significantly high titre values in cervicitis sera at 1:100 (P=0.0002***),1:1000 (P=0.0154*) and 1:10,000 (P=0.0017**); b) OD values for anti-IncC antibodies showed statistically significant high titre values in cervicitis sera at serial dilutions 1:100 (P=0.0178*) and 1:1000 (P=0.0179*).

Figure 5. Antibody subtype titres against CT Incs in patients' sera.



Quantitation of anti-IncB (5a) and anti-IncC (5b) specific IgG subtypes in CT-positive and CT- negative sera. Median values of IgG_1, IgG_2, IgG_3 and IgG_4 were compared between CT-positive and CT-negative sera. *** represents P < 0.0001, *i.e.* highly significant or ** represents P < 0.05, *i.e* significant. IgG subclass titre values for GST alone were very low in both CT-positive and CT- negative sera (data not shown).

Table 1. Cytokine levels (pg/mL) in serum samples of CT-positive, Inc-positive women diagnosed with cervicitis or PID/ infertility

	MOMP+ve , IncB +ve Cervicitis (n= 38)	MOMP + ve,IncB +ve PID/ Infertility (n=21)	P value	MOMP+ve , IncC +ve Cervicitis (n= 45)	MOMP + ve,IncC +ve PID/ Infertility (n=17)	P value
IL-1ß	16.9 (7.8 -26.7) [a,b]	21.2 (9.7 -36.2)	0.0158	22.7 (12.3 -32.3)	23.3 (1.6.1 -30.7)	*NS*
IFN-γ	83.4 (66.2 -132.4)	27.8 (16.8 -54.3)	<0.0001	93 (69.5 -138.7)	24.2 (12.6 -44.3)	<0.0001
TNF-α	54.85 (26.3 -72.1)	31.8 (16.8 -53.7)	<0.0001	61.1 (31.6 -78.1)	24.3 (12.6 -44.3)	<0.0001
IL-10	63.14 (37.4 -78.9)	81(36.8 -91.2)	0.0231	57.3 (35.7 -76.3)	79.4 (45.5 -99.6)	0.038
IL-12	79.3 (45.6 -89.5)	47 (36.8 -61.4)	0.0059	68.9 (57.4 -77.9)	48.2 (32.1 -65.3)	0.0043

[a]Data are median cytokine levels in picograms per millilitre unless otherwise noted.
[b]Numbers in parentheses denote range.

against IncB were higher than those against anti-IncC in these sera at dilutions 1:100 ($p = 0.0028$) and 1:1000 ($p = 0.0189$); however, MOMP titres were the highest, suggesting that MOMP is more immunogenic than Incs.

There was significant positive correlation between antibodies against Incs and MOMP in CT-positive sera suggesting thereby that Incs are expressed simultaneously during chlamydial infection and also with established infection. It has been reported by Bannantine *et al.*, that *C. psittaci incB* and *incC* are co-transcribed in an operon and that there is identical arrangement of these genes in the CT genome with homologous sequence identity matches [20]. Thus, as seen in our data, simultaneous expression of both Inc proteins could be attributed to their respective genes being activated simultaneously.

In a bid to find an association between disease pathology and seroprevalence of Incs, the present study found high IncB and IncC antibody titres in cervicitis patients in comparison to those with PID. Significantly high titres of antibodies against IncB in CT-positive cervicitis sera in comparison to PID/infertility was detected, at up to a dilution of 1:10,000, whereas that of IncC was detectable at up to a 1:1000 dilution of the same. Differential immunogenic properties of inclusion proteins and their involvement in particular disease pathologies could be a result of multiple factors such as subcellular localization, cytoplasmic exposure, and spatial arrangements on the inclusion membrane or within the inclusion needs further research.

We also found significant levels of anti-incB and anti-incC IgG_2 and IgG_3 isotypes in CT-positive sera in comparison to IgG_1, IgG_3 and IgG_4 in CT, indicating that there is predominant Th_1 response. Since the relation between the production of IgG subclasses and T helper cytokines in humans is not as well defined as it is in the mouse, our findings regarding a Th_1 mediated protection can only be confirmed by measurement of Th_1 related cytokines from PBMCs isolated from these patients and stimulated with CT inclusion proteins B and C. *Pal et al.* had previously reported that to establish the protective role of sera IgG_{2a} antibodies in 3 strains of mice against a chlamydial genital challenge, IFN-γ and IL-4 responses were monitored and correlated to the initial findings [31,32]. Our data showed significantly higher levels of pro-inflammatory (TNF-α, IL-12 and TNF-α) and inflammatory cytokine IFN-γ in Inc-positive sera from cervicitis sera with respect to PID/Infertility. This observation suggests that there is a protective role of infection clearance at the systemic level within infected host cells. Thus inflammatory cytokines secreted by the infected immune cells may play an essential role in immunity and in the immunopathogenesis of chlamydial infection by initiating and regulating inflammation as well as the immune responses.

The unavailability of three-dimensional structures of IncB and IncC has hampered our understanding of the spatial arrangement of epitopes and other probable active pockets and domains critical in generating antibodies to these proteins within infected cells. Structural analyses of these proteins would also elucidate whether these epitopes or other unidentified ones are candidates for epitope mimicry and form mimetopes during their interactions with infected host cells. Further characterization of B cell epitopes of these proteins will help in our understanding of whether these

peptides have a role in humoral responses generated by these proteins.

In conclusion, to the best of our knowledge this is the first study from India on the detection of CT infection by measuring antibody titres against chlamydial inclusion membrane proteins IncB and IncC in sera of CT-infected patients. Further, this study also aimed to correlate severity of chlamydial infection with antibodies against CT Incs and found higher titres of anti-Inc antibodies in CT-positive cervicitis patients, which can be seen as an indication of their role in initial infection rather than in established disease pathologies such as PID or infertility.

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