Original Article

Current *Chlamydia trachomatis* Infection, A Major Cause of Infertility

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Abstract

Background: In India, the impact of current *Chlamydia trachomatis* (*C. trachomatis*) in reproductive health remains a neglected area of investigation. The present study evaluates if current Chlamydia infection is associated with any clinical complication that needs the attention of clinical investigators.

Methods: In this cross-sectional study, we enrolled 896 women attending the Gynecology Out Patient for the detection of *C. trachomatis* infection. Polymerase chain reaction was used to diagnose current *C. trachomatis* infection and ELISA for past infections. Bacterial vaginosis, Candida and Trichomonas were screened. The results of symptomatic and asymptomatic groups were compared. The data was analyzed using Epi Info version 6 and "Z" test. A probability value of $p \leq 0.05$ was considered as significant.

Results: Statistical analysis revealed significant association between current *C. trachomatis* infection with infertility when comparing infected fertile (18.6% *vs.* 9.4%, odds ratio: 2.19, $p<0.0005$) and uninfected infertile women (45.6% *vs.* 27.3%, odds ratio: 2.24, $p<0.0001$). Average infection rate was 12.1%, highest in women with infertility (18.6%) or with ectopic pregnancy (25%). Significant proportions of infected women with infertility ($p<0.01$) or with recent pregnancy ($p<0.001$) were asymptomatic. Follow up of infected women who became negative after treatment [28 women from infertility group and 9 women with recurrent spontaneous abortion (RSA)] revealed live birth in 8 (21.6%) women within one year, 4 with infertility and 4 with RSA.

Conclusion: Study findings suggest association between current *C. trachomatis* infection and infertility. Absence of signs and symptoms associated with this infection highlights its diagnosis in women with a history of infertility and RSA for their better management, as revealed by live births with one year of follow up.

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Introduction

Diagnosis, treatment and prevention of sexually transmitted Chlamydia infection has become an important public health priority especially, by strong evidence linking this infection with HIV transmission (1). Consequences of *Chlamydia trachomatis* (*C. trachomatis*) infection are more damaging to the reproductive health of women than to men. A number of clinical conditions like mucopurulent endocervicites, endometritis or salpingitis have been attributed to this infection. The potentially serious sequelae of cervical infection with *C. trachomatis* includes infertility, ectopic pregnancy, pelvic pain and recurrent pelvic inflammatory diseases (PID) (2–4). However, all the infected individuals do not develop such complications or symptoms, as only a frac-

tion get upper genital tract infection and a subset of them manifest complications leading to infertility and ectopic pregnancy (5, 6). Direct and indirect costs of chlamydial infections are substantial, justifying more attention and a stronger multidisciplinary approach. Cates and Wasserheit reviewed a large number of studies showing statistically significant association between tubal factor infertility, spontaneous abortion (SA) and ectopic pregnancy with previous systemic chlamydial infection identified by the presence of *C. trachomatis* specific antibody (7). Reports are also available on the prevalence of current *C. trachomatis* infection in women with different clinical conditions like infertility and genitourinary complaints (8-10); however, there is not much reports on its association with infertility or related clinical complications. Evaluation of present infection with the aforesaid types of manifestations could help in treatment. In India, the clinical manifestations or sequelae associated with current *C. trachomatis* infection, is yet to be considered as a major health problem and clinician rarely refer any subject for its diagnosis. Hence, the present study aims to assess whether current *C. trachomatis* infection is associated with any complications in Indian women that needs to be highlighted for its clinical investigation.

Methods

Subjects: In this cross-sectional study, we enrolled women attending the Gynecology Out Patient Department (OPD) of Seth G.S. Medical College and King Edward Memorial (KEM) Hospital, Parel, Mumbai, between 2003 to 2009. The group comprised of women with histories of recurrent spontaneous abortion (RSA, n=143), infertility (n=264), symptoms and signs of lower genital tract infections (LGTI, n=213), pregnant women (n=174) attending the antenatal care (ANC) unit, as well as those who had no symptoms and signs of any infection (asymptomatic controls, n=102) but came for family planning advice.

Ethics Committees of the institute, as well as of KEM Hospital approved the study. Each woman was informed about the study and written consent was obtained from all the women before enrollment.

Specimens: The clinician team did a routine gynecological per speculum examination to record signs of infection and collected endocervical and vaginal swab specimens. First, endocervical swab (Hi-media Laboratories Pvt. Limited, Mumbai, India) specimens were collected in a sterile container with 1ml PBS (pH=7.5) for immediate processing to detect *C. trachomatis* infection, while second specimens were stored in dry sterile vials at -20 *℃* for confirmatory tests, if required. Vaginal specimens were also collected from the posterior fornix using a wooden spatula for the diagnosis of bacterial vaginosis (BV) using Nugent's scoring system for Gram stain smears (11) and for the detection of trichomonas and candida by wet mount. Blood specimens collected from the women were used for antibody test using commercially available ELISA kit (Novatech Immuno-diagnostica, GMBH).

Adequacy of specimens: In order to check specimen adequacy, each cervical specimen in PBS was vortexed for 30 *s*, the swab was squeezed and 10 *µl* of specimen was examined under microscope to see the presence of epithelial cells. Four to five epithelial cells per high power field was considered as an adequately collected specimen for further processing.

Signs and symptoms of reproductive tract infections: Severely eroded cervix with hypertrophic cervical erosions and a mucopurulent endocervical discharge or leucorrhoea were recorded as signs while burning micturation and pain in the abdomen reported by the women were recorded as symptoms.

Extraction of DNA: DNA was isolated from cervical specimen using a rapid non-enzymatic method. The cells were pelleted and resuspended in Tris-$MgCl_2$-KCl buffer (pH=7.4) and treated with 10% sodium dodecyl sulphate at 55 *℃* for 10 *min* to lyse the cells. The proteins were precipitated using saturated sodium chloride solution. DNA was precipitated by 100% ethanol and eluted in Tris EDTA buffer (12). The quantity and quality of DNA was estimated spectrophotometrically and by loading an aliquot of DNA on 0.8% agarose gel. As an internal control PCR for beta-globin gene was also performed for each sample to rule out the presence of inhibitory factors in the extracted specimens.

PCR for diagnosis of C. trachomatis: PCR was performed on extracted DNA using primers designed from the conserved region of MOMP gene of *C. trachomatis* with sense primer: 5' GCC GCT TTG AGT TCT GCT TCC 3' and anti-sense primer: 5' GTC GAA AAC AAA GTC ACC ATA GTA 3' to amplify a 180 *bp* DNA fragment common to all serotypes (13). The reaction was carried out in a volume of 50 *µl*. It contained primers (0.5 *µm*

each), 0.2 *mM* dNTP's, PCR buffer (10 *mM* Tris buffer; pH=9), 1.25 units of Taq polymerase, 10 *µl* of DNA specimen and the volume was adjusted with sterile distilled water. Positive and negative controls were also run in each experiment. Reaction was performed in a thermal cycler (Perkin Elmer 2400) as per the following protocol: initial denaturation was done for 5 *min* at 94 *°C*. This was followed by 35 cycles of 30 *s* each of denaturation at 94 *°C*, annealing at 55 *°C* and extension at 72 *°C* for 1 *min*. The final extension step was carried out at 72 *°C* for 5 *min*. The amplified products were run on 2% Agarose gel, observed under a UV transilluminator while the results were being documented. Presence of 180 *bp* repeat sequences in positive control specimen and its absence in the negative control indicated reaction had been completed satisfactorily. Presence of 180 *bp* repeat sequences in other clinical specimens indicated presence of *C. trachomatis* infection. Further confirmation of these amplified products was carried out using specific *C. trachomatis* probe in Southern hybridization (14). Probe was prepared using PCR dig-labeling kit (Roche diagnostics). Standard protocol for Southern blotting was followed for transfer of PCR products to a nylon membrane, which was then processed for hybridization using a generic probe. Instruction manual was followed to detect the probe complex using Dig-luminescence detection kit (Roche diagnostics).

Detection of C. trachomatis IgG antibody: Commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to detect *C. trachomatis* specific IgG antibody (NovaTec Immunodiagnostica, GMBH). In brief, microtitre wells precoated with *C. trachomatis* antigens were incubated with serum specimen at a 1:100 dilution so that any corresponding antibodies present in the serum would bind to the antigen to form complexes. After washing the wells to remove all unbound sample material, horseradish peroxidase (HRP) labeled anti-human IgG conjugate was added which would bind to captured Chlamydia specific antibodies. The immune complex formed by the bound conjugate was visualized by adding tetramethylbenzidine (TMB) substrate, which gives a blue colored reaction product.

After terminating the reaction using a stop solution (Sulphuric acid, 0.2 *mol/l*), the absorbance of the end product, which is yellow in color, was read at 450 *nm* using an ELISA plate reader (*µ* Quant, Bio-Tek Instruments Inc.). The intensity of this product is directly proportional to the amount of Chlamydia-specific IgG antibodies in the specimen. The specimens with O.D. higher than the cut-off value (0.250–0.900) were considered positive for Chlamydia-specific antibodies and used as an indicator of past Chlamydia infection. Each positive sample was again confirmed using another serum aliquot of the same participant. The results were found to be reproducible.

Follow up of C. trachomatis positive cases: Counseled each enrolled women to come back to take the report. Those found to be infected with any of these infections were treated by the clinician.

Statistical analysis: Statistical analysis using Epi Info version 6 software for Chi-squares (χ^2) test was applied to study the association between *C. trachomatis* infection with the clinical manifestations. The test of significance for proportion between different groups was carried out using "Z" test. A probability value of $p \leq 0.05$ was considered as significant.

Results

Study subjects: Eight hundred and ninety-six women were tested for current *C. trachomatis* infection by PCR. The participants were between 16 to 45 years old with a median age of 29 *yrs*, and an interquartile range (IQR) value of 10. They belonged to middle socio-economic groups and their personal history did not reveal any high risk behavior. The number of women in asymptomatic control group, as well as those in groups with different clinical histories like RSA, infertility, with lower genital tract infection (LGTI), pregnant women from antenatal care (ANC) centers, their age and the infection rate in each group is presented in table 1.

In the RSA group, there were 58 women with 2 pregnancy losses (2SA), 77 women with more than 3 pregnancy losses (>3SA) and 8 with ectopic pregnancy. In the ANC group, the gestational period of the pregnant women varied from 2 to 4 months. There were 108 (12.1%) women with current *C. trachomatis*, indicating the prevalence of this infection in the study population.

Presence of other reproductive tract infections and past C. trachomatis infection: Of the 108 *C. trachomatis* infected women, 4 (3.7%) had concomitant BV while 1 (0.9%) had concomitant *Candida albicans*. In the rest of participants (n=788), *C. trachomatis* specific antibody was present in 14 women, one woman had both the antibody and the antigen. Eighty women had other infections such

Table 1. Defined groups of participant (count and age) and *C. trachomatis* infection rate

Clinical groups	Participant (n=896)		Age in years		Ag		*C. trachomatis*		Other Infections		
							Ab	Ag+Ab			
	N	(%)	Range	Median	N	(%)	N (%)	N (%)	BV	*Candida*	*Trichomonas*
Asymptomatic	102	11.4	18-40	30	2	(1.96)	0 (0.00)	0 (0.00)	6	2	0
LGTI	213	23.8	18-40	30	20	(9.39)	0 (0.00)	0 (0.00)	6	15	5
RSA (n=143, 15.38%)											
2 SA	58	6.5	20-43	26	3	(5.2*)	--	--	9	5	0
>2 SA	77	8.6	20-40	28	8	(10.4*)	--	--	3	1	0
Ectopic pregnancy	8	0.9	26-38	31.5	2	(25*)	--	--	0	0	0
Infertility	264	29.5	18-40	26	49	(18.6)	6	0 (0.00)	18	2	2
ANC	174	19.4	19-40	26	24	(13.8)	0 (0.00)	1	1	10	0

*p<0.001,

Notes: Asymptomatic=Healthy women without any sign or symptoms of any infection or disease; LGTI=Lower genital tract infections; RSA=Recurrent spontaneous abortion; Infertility=Women unable to conceive after two years of cohabitation with husband; ANC=Antenatal cases or pregnant women; SA=Spontaneous abortion

as BV, Candida or Trichomonas infections and the related infection rates were 14.5%, 4.3% and 0.9%, respectively. For further analysis, these women with *C. trachomatis* antibody (n=15), as well as those with other infections (n=80) were excluded. Hence, there were 693 women without any infection, who were taken into consideration for comparative analysis (Table 2).

Sequelae, symptoms and signs associated with current C. trachomatis: Infection rate varied from 1.96% to 25.0% among the different groups of participants (Table1). Among the RSA subgroup a significant (p<0.001) proportion of women with ectopic pregnancy (25%) and with more than 2 spontaneous abortions (10.4%) had this infection, compared to women with 2 spontaneous abortions (5.2%). *C. trachomatis* infection rate was significantly low in the group of women with children or expecting a child such as asymptomatic controls (n=102), LGTI (n=213) or in ANC (n=174) groups, {9.4% (46 of 489); odds ratio: 2.19, p<0.0005)} when compared to infected infertile women (18.6%, 49 of 264). Comparison of clinical manifestation of women with only current *C. trachomatis* infection (n=103) with that of uninfected women (n=693) revealed significant association of *C. trachomatis* infection with infertility (45.6% *vs.* 27.3%, p=0.0001; Table 2). Another significant observation was the absence of any symptoms or signs on per speculum examination in infertile (64% *vs.* 36%, p<0.01) and pregnant (79.2% *vs.* 22.8%, p≤0.001) infected women, indicating asymptomatic nature of this infection (Figure 1).

Age associated with current C. trachomatis infection: *C. trachomatis* infection was highest (21.8%) among women 20 years old or younger, though not statistically significant, and lowest in 21–25 year old age group. The infection rate again showed an increasing trend in women above 26–40 years of age.

Table 2. Frequency of clinical manifestations with or without current *C. trachomatis* infection in women who did not have any other infection and their treatment outcome

Women	*C. trachomatis*											
	Positive		Negative		OR	χ^2	p	Treatment given	Treatment outcome *C. trachomatis*		Live birth after treatment	Loss to follow up
	N=103		N=693						Negative	Positive		
	N	(%)		(%)								
Infertility	47	45.6	189	27.27	2.24	13.3	0.00014	42	28	2	4	12
RSA	11	10.7	106	15.29	0.66	1.63	0.217	11	9	2	4	-
ANC	24	23.3	138	19.91	1.22	4.47	0.425	8	not known		8	-
LGTI	19	18.4	168	24.24	0.71	1.13	0.195	9	9	--	--	--
Asymptomatic control	2	1.9	92	13.28	-	9.67	0.0002	2	2	--	--	--
Total	103	-	693	--	--	--	--	72	48	4	16	12

Notes: Infertility=Women unable to conceive after two years of cohabitation with husband; RSA=Recurrent spontaneous abortion; ANC=Antenatal cases or pregnant women; LGTI=Lower genital tract infections; Asymptomatic controls=Healthy women without any sign or symptoms of any infection or disease

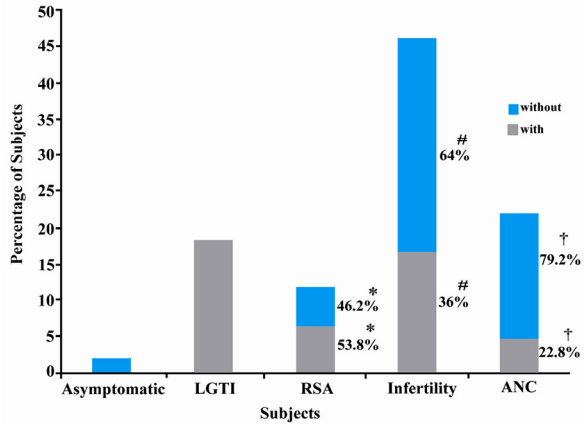

Figure 1. Sequelae associated with *C. trachomatis* infection, with or without symptoms and signs
*p>0.05; #p<0.01; †p<0.001, LGTI=Lower genital tract infection; RSA=Repeated spontaneous abortion; ANC= Antenatal case. Percentage of subjects=Percentage of infected women in each group of women with different manifestations

Association of current C. trachomatis infection with vaginal pH/Microscopic analysis (>10PMNs)/Colour of swab/Bleeds on touch: Among the *C. trachomatis* infected women, (20.2%) had high pH (≥5), (47.7%) had more than 10 polymorphonuclear leukocytes (PMNs) in their specimens, 2.7% had yellow/grayish coloured discharge and 7.3% had blood on swab or bled during collection of specimens.

Follow up of C. trachomatis positive cases: Seventy-two women with *C. trachomatis* infection came for the report, which were subsequently treated. Follow up record on 60 women was available only for one year. Thereafter, they could not be traced due to several reasons. In ANC group, 8 of 24 *C. trachomatis* positive pregnant women came for follow up and were treated. No further testing was done in these pregnant women after completion of treatment in accordance with the clinician advice to avoid any risk during pregnancy. These eight women had live births. Result of follow up in other groups revealed that four of the 52 infected individuals were positive even after treatment. There were eight live births in these groups following treatment (Table 2). Women with other infections such as BV, Candida and Trichomonas were also treated as per hospital routine procedure.

Discussion

Results revealed statistically significant association between current *C. trachomatis* infection with clinical manifestations or sequelae like infertility in women in Mumbai, India. Women with confounding variables such as other abnormalities or infections were excluded from comparisons. In our study, we excluded women positive for *C. trachomatis* antibody from analysis as presence of *C .trachomatis* antibody is known to be associated with tubal factor infertility, spontaneous abortion and ectopic pregnancy (7). A recent report in Ghanaian women, also highlighted the presence of Chlamydia-specific IgG (39%) and IgA (14%) antibodies indicating previous *C. trachomatis* infections among women with primary or secondary infertility compared to current infection (2.4%) (15). Further, *C. trachomatis* infected women with co-infections were also excluded from the study so that a direct correlation could be made between current *C. trachomatis* infection with its clinical manifestations. In the present study, comparative analysis between women with or without *C. trachomatis* with different types of clinical manifestations, showed a statistically significant association between current *C. trachomatis* infection with infertility.

The average infection rate was 12.1%. This high rate of infection might be due to the inclusion of women with specific clinical history like infertility, ectopic pregnancy, as well as women with more than two spontaneous abortions. Previous studies in the local population have shown a low infection rate among women with infertility (2.5%) which might have been due to the use of less sensitive techniques like ELISA (16). Our previous study using the same method (ELISA) also showed a similar infection rate (1.7%) in asymptomatic controls, while the present rate (18.6%) of infection in women with infertility using PCR was high compared to 14.3% of women with infertility published earlier using ELISA (17). Another study from Mumbai reported high rates of infection (23.2%) in female sex workers using ELISA (18), indicating presence of this infection in the local population. Moreover, women with infertility and recurrent spontaneous abortions might be more sexually active to conceive leading to high infection rates. Other studies in women from northern Indian also revealed similar high infection rates (27%, 20 of 74) in women with primary infertility as detected by culture or antigen test (8). High prevalence rate (43.1%) of *C. trachomatis* was also seen in women (n=430) with genitourinary complaints, even among the slum dwellers (15.3%, n=53) compared to 9.39% observed in our women with LGTI (10). Report also revealed high rates of Chlamydia infection in women with infertility (36%, n=169), compared to our observation of 18.6% (9). Provision of free

treatment and access to health care system might be responsible for the comparatively low infection rate seen in this western region of the country.

Significant proportion of women with ectopic pregnancy and more than two spontaneous abortions had current *C. trachomatis* infection, which might be the etiology for the aforesaid disorders as reported earlier (7, 19). However, these reports suggest association of *C. trachomatis* antibody or its past infections with these types of manifestations, whereas our results showed its association, *i.e.*; one fourth of ectopic pregnancies with current *C. trachomatis* infection, supporting the recent review which attributes one- third of ectopic pregnancies to chlamydial infection (2).

Age-wise distribution of study population with *C. trachomatis* infection revealed that a high proportion of women younger than 20 years of age had this infection, which is in harmony with other reports that Chlamydia infection rates are inversely related to age (5–7, 20, 21). In the present study, a unique trend between infection rate and age was observed.

This infection was mostly asymptomatic; women only came to the clinics when they developed complications such as signs and symptoms of lower genital tract infection, experienced repeated pregnancy loss, and had infertility at later age; thus, present observation revealed an increasing rate of infection with age.

A year of follow up of the treated women was followed by the pregnancy and subsequent live birth in four women with infertility and four women with RSA indicating the association of this infection for such types of manifestations.

Asymptomatic nature of these manifestations also correlated with earlier reports (22, 23). Some studies have shown that a count of <10 PMNs per high power field was defined as predicting absence of gonococci and *C. trachomatis* (24, 25) but only 47.7% of the infected women from the study group had a count of >10 PMNs/hpf. Absence of increased number of PMNs in the rest might be associated with asymptomatic nature of disease manifestations in these women.

We could not establish the cause of high pH of vaginal secretion in 20% of the *C. trachomatis* infected cases as only 3.7% had BV infection. Hence, our attempt to correlate *C. trachomatis* infection with any changes in vaginal pH, any changes in the colour of swab collected or bleeding while collecting specimen was futile.

In *C. trachomatis* negative women, the rate of other infections like BV, *Candida* and *Trichomonas* correlated well with a previous study conducted in Mumbai where the infection rate of BV, Candida and Trichomonas were, 13% (58/446), 0.9% (4/446) and 0.5% (2/446), respectively (26). Infection such as candidiasis (4.3%) was expected to be more common in Mumbai in view of poor and unsatisfactory housing conditions under which many of them lived but this condition was not observed. Even existence of common RTIs along with *C. trachomatis* infection was observed to be low in this clinic based prevalence study.

These findings could suggest, statistically significant association between current *C. trachomatis* infection with infertility and immunity to infection which might be correlated to sperm rejection in women leading to infertility.

The limitation of the study was follow up of enrolled women only up to one year. The enrolled women could not be contacted due to frequent change of their phone number.

Absence of signs and symptoms in significant proportions of currently infected women, as well as high infection rate in the younger age group emphasizes the need for *C. trachomatis* diagnosis. Current *C. trachomatis* infection could be involved in the etiology of infertility and its treatment will help in positive pregnancy outcomes.

Conclusion

Study findings suggest, statistically significant association between current *C. trachomatis* infection with infertility. Absence of signs and symptoms associated with this infection highlights the need for its investigation in women with a history of infertility and RSA for their better management, as revealed by live birth with one year of follow up.

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Conflict of Interest

There was no conflict of interest in this article.

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