Chapter 6

Discussion
6. DISCUSSION

6.1 GENITAL CHLAMYDIAL INFECTIONS IN THE APPARENTLY HEALTHY POPULATION OF TAMIL NADU

The role of *C. trachomatis* as an important STD agent is well recognized throughout the world. The prevalence and distribution of genital chlamydial infections in the general population is however poorly understood. While very few studies have been carried out in other countries (Jonsson *et al.*, 1995; Van Valkengoed *et al.*, 2000; Klausner *et al.*, 2001; Turner *et al.*, 2002), studies have not taken place in India to understand the epidemiology of genital chlamydial infections in the community population. The present pilot study, by ‘Probability Proportional to Size’ population-based cluster survey technique, assessed the prevalence of these infections in the apparently healthy adult community population of sexually active age in Tamil Nadu.

While, PCR was used to study the prevalence of active infections, serology using IgM ELISA was done to estimate the prevalence of recent/ongoing infections. The prevalence of chlamydial infections by antibody serology (IgM ELISA) was 2.4% (95% CI 1.6%-3.2%). The overall prevalence of active genital infections due to *C. trachomatis* in the apparently healthy population of Tamil Nadu as determined by urine PCR was 1.1% (95% CI 0.5%-1.7%). A population-based study in Sweden (Jonsson *et al.*, 1995) reported a prevalence of 2.7% using culture, while the
seroprevalence (IgG) was 24.7%. No other combined PCR and seroepidemiologic population-based studies are available.

The PCR determined prevalence in the present study is relatively low compared to the population-based reports from other countries. In a similar probability household sample survey in Baltimore, USA, the estimated prevalence of genital *C. trachomatis* infections was 3% (Turner et al., 2002). A study done in The Netherlands reported a prevalence of less than 3% in the general population (Van Valkengoed et al., 2000). Other community-based studies, reported higher prevalence rates up to 7% (Tabrizi et al., 1997; Colvin et al., 1998, Munk et al., 1999). Combining both anti-chlamydial IgM antibody and *C. trachomatis* DNA positivity, the overall positivity in the present study was 3% (95% CI 2.2%-3.8%). This was in accordance with those prevalence studies done in northern California and Baltimore, which have shown the prevalence rates to be 3.2% and 3% respectively (Klausner et al., 2001; Turner et al., 2002).

The present study observed almost an equal distribution of genital *C. trachomatis* infections in male as well as female populations (1.2% and 1.1% respectively). However, the seroprevalence by IgM ELISA was significantly higher in females (3.3%) compared to that of males (1.3%). The reason behind this gender specific difference remains unclear. It may be assumed that the exposure to chlamydial infections might have been mostly primary in females rather than repeated. However, an IgG antibody determination was not done to hold such an assumption to be valid. In the present study, positivity patterns by serology and PCR
showed slight differences with respect to the age groups. The overall age specific *C.trachomatis* positivity pattern by IgM ELISA as well as PCR has suggested a declining trend in the infection rate in the later years of sexually active age (36-45 yrs) in the general population.

The seroepidemiology in the three districts of Tamil Nadu showed a higher seroprevalence in Tanjore compared to Ramnad and Dindigul. The different rates of infection across the districts may reflect differences in the socio-demographic factors related to disease transmission. It is possible that variations in the prevalence rates could occur with relation to geographical regions. India, being a country with cultural and social diversities between the states and within the states, these diversities may have resulted differences in the prevalence rates of this sexually transmitted infection. Considerable variation in the prevalence could be expected among many communities within Tamil Nadu itself. The exploration of district-specific correlates of *C.trachomatis* infection was beyond the scope of the present study. The prevalence of active *C.trachomatis* infection was seen higher in Dindigul district compared to that of the other two districts. However, this apparent difference did not show statistical significance. Our data corroborates with that of the randomized population study done in northern California (Klausner *et al.*, 2001), which observed differences in the infection rates among the three regions of San Francisco Bay Area (Alameda, San Francisco, and San Mateo).

Most of the earlier community-based studies in the international scenario were limited to female populations since female genital tract infections due to
*C. trachomatis* are more often associated with discernable reproductive tract sequelae. Moreover, screening asymptomatic males required invasive means of sampling procedures. With the advent of nucleic acid amplification testing with noninvasive urine samples, large-scale studies in community-based settings became possible. For women, other samples (for example, vaginal flush and vaginal swabs) have also been evaluated (Stary *et al.*, 1997; Weisenfeld *et al.*, 2001). These specimens have been shown to be more sensitive than urine for *C. trachomatis* detection in females.

A recent population-based study conducted in Britain (Fenton *et al.*, 2001) has shown a higher prevalence of chlamydial infection in males (2.2%) compared to 1.5% in females and emphasized the absolute necessity for including men in the screening programmes. While there was no significant difference in the prevalence rates (by PCR) with males and females in the present study, our observation that most of the infected men (85.5%) were asymptomatic supports this proposition.

In view of the low PCR positivity for *C. trachomatis* with a higher rate of seropositivity, one could postulate that infections would have cleared off in a substantial proportion of the IgM positive cases. While a positive PCR test could represent a likely active infection, a positive IgM serology may also reflect recent infection. It is unknown under which condition *C. trachomatis* infection causes seroconversion or for how long the detectable antibodies persist after infection (Jonsson *et al.*, 1995). It may be assumed that PCR negativity in the IgM antibody positive cases could also be due to some proportion of acute, silent upper genital tract infections with *C. trachomatis*, which is often difficult to demonstrate by direct tests.
The poor correlation observed between IgM ELISA and PCR reflect the difference in the detection of different targets i.e., the presence of antibody versus DNA. In order to avoid recognized problems of cross reactivity with other *Chlamydia* species, especially *C.pnuemoniae*, we have used a kit to detect specific IgM antibodies against *C.trachomatis*. While PCR was positive in only 5 of the 45 (11.1%) IgM positive cases, positivity was seen in 11 (0.8%) of the IgM negative cases by PCR. It is known that PCR could detect CT infections at the early stages, even before seroconversion. It is also understood that IgM response is seen in primary infections only. The PCR positivity in the IgM negative group could be also due to some repeat or reinfections.

In this large community-based survey, genitourinary symptoms were observed in 45.3% of the total subjects analysed. Vaginal discharge contributed to the high prevalence of genitourinary symptoms in the community. We observed a poor correlation between laboratory proven chlamydial infections and the symptoms. Other reproductive tract infections or STDs that were not measured in this study could have contributed to these symptoms. Our data support studies (Cecil *et al.*, 2001), which have shown that screening on the basis of symptoms alone would miss the majority of *C.trachomatis* infections.

It was seen that 68.8% of the PCR positive cases in our study did not experience any of the symptoms. This highlights that asymptomatic *C.trachomatis* infection is prevalent in the general population of Tamil Nadu. A population-based survey of an adult rural South African community (Colvin *et al.*, 1998) has shown that
all the detected infections were asymptomatic. Asymptomatic persons are less likely to seek medical care, are potentially at risk for developing complications, and may represent a large reservoir of infection for continued transmission. Our data suggests that there is a significant burden of undiagnosed asymptomatic genital chlamydial infection in the population. It is of great concern that these asymptomatic infections could contribute to the silent transmission of the disease in the community.

Many studies have shown association of *C. trachomatis* infections with young age (Munk *et al.*, 1999; Morre *et al.*, 1999b; Burstein *et al.*, 2001). In this population-based study, both seropositivity and PCR positivity were not significantly associated to young age. This is at variance with the general idea that *C. trachomatis* infections are more often associated with age less than 25 years. The findings of this community-based study should be viewed in the Indian context. Even though, the risk of acquiring chlamydial infections is quiet high in young age, it needs to be emphasized here that the epidemiology of a sexually transmitted infection is conditioned by the sexual practices, which in turn are specific to the cultural practices of the community population. Thus, our findings may have contrasted most of the western studies, which have shown the association of *C. trachomatis* infection with young age.

*C. trachomatis* positivity was not found associated with any of the known risk factors in the present study. This might be attributed to the fact that the study subjects represented the general population and to the low risk behaviour observed. It is presumed that risk behaviour and STIs like genital chlamydial infections may be more
localized in certain high-risk groups in the community. The ‘medical camp approach’ adopted for the sample collection was helpful in attaining the maximum representation from all the strata of the population. An underestimation of prevalence is likely, if those who did not turn up at the medical campsite had high-risk behaviour. In western countries, the use of mailed home-obtained urine specimens has become a popular tool for screening the asymptomatic population in the community (Ostergaard et al., 1998, Morre et al., 1999a; Stephenson et al., 2000). However, in Indian situations, this may be a difficult proposition because of inadequate infrastructure and poor educational status in the low socio economic strata of the general population.

Although random population-based studies are costly, time consuming and difficult to conduct, they provide an important means of estimating of disease burden in the population (Klausner et al, 2001). Report based estimation and prevalence monitoring for genital chlamydial infections have been rarely attempted in India, as costly diagnostic facilities required for C.trachomatis infections are not available in most of the STD referral settings due to resource constraints. Moreover, these methods may underestimate the prevalence in the population at large. The present population-based study is the first of its kind in India. By adopting appropriate screening methods and probability sampling from the general population, the present study permits generalizations about the prevalence and epidemiological pattern of genital chlamydial infections in Tamil Nadu. The baseline data from the present study is useful in the programmatic implication of targeted intervention to control these sexually transmitted infections. Given the importance of early treatment to prevent complications, selective screening has been suggested by others as an
efficient strategy for screening in low prevalence populations (Grun et al, 1997). Our results suggest that an effective selective screening strategy for a low risk population is difficult to achieve, if not impossible. Although screening and treatment are critical components of any strategy to reduce the genital infections with *C. trachomatis* and their adverse sequelae, the opportunity for primary prevention cannot be overlooked. Screening should be augmented by effective behavioural, sexual risk reduction interventions.

6.2 GENITAL CHLAMYDIAL INFECTIONS IN SYMPTOMATIC STD PATIENTS.

6.2.1 Prevalence

Even though, genital infections caused by *C. trachomatis* and its important sequelae are well documented in the developed countries, these infections are not well studied in developing countries like India even in clinic settings. There is a paucity of data from India about the prevalence of genital chlamydial infections in symptomatic STD patients. This is probably due to the lack of costly diagnostic facilities, which are required for *C. trachomatis* in most of the STD referral centers. Except few, many of the previous studies have used less sensitive conventional methods like antigen detection or serology. A single report of culture has shown a prevalence of 41% in the symptomatic women attending gynaecological clinic (Mittal et al., 1993). By using PCR, a positivity of 50% (25/50) in women STD patients was reported (Gopalakrishna et al., 2000). More recently, a study done by Singh et al., (2002) has
shown a prevalence of 43.1% in symptomatic women attending a gynaecology clinic in Delhi.

The prevalence of *C. trachomatis* infections observed in women in our study was 27.5%. This is well consistent with a recent study (Divekar et al., 2000), which has shown a prevalence rate of 27.2% in women attending a STD clinic in Mumbai. There is virtually no data from South India about the genital infections due to *C. trachomatis* in STD patients. The only study, so far, was that of Alexander et al., (1993), which observed a low prevalence rate of 3.3% in pregnant women. Most of the previous studies in India were focused on women. Relatively little information is available about the prevalence of genital chlamydial infections in men attending STD clinics in India. The higher prevalence of 34.9% in men observed in the present study indicates the need for screening all the men attending STD clinics. A recent report from Mumbai (Mania-Pramanick et al., 2001) has shown an infection rate of 33.3% in male partners (n=15) of the infected women who attended gynaecology and infertility clinics. It is important to consider that undiagnosed chlamydial infections in men could facilitate sustained transmission of infection to their female partners. It is therefore emphasized that efforts to control chlamydial infection that ignore men will ultimately fail (Taylor-Robinson and Renton, 1999) and hence screening for *C. trachomatis* should be done for both men and women attending STD clinics.

The overall prevalence of proven *C. trachomatis* infections in our study was 30.8% (95% CI 23.5%-39.1%). The observed prevalence is relatively high in comparison to the rates of 5-15% found in similar STD clinic populations of other
countries in recent years (Hook et al., 1992; Hart, 1993; Crowley et al., 1994; Van Duynhoven et al., 1997). Our results indicate that genital chlamydial infections are prevalent significantly in STD patients in this part of the country. The risk variables may most likely be over represented in symptomatic STD patients compared to those high-risk groups in general population. Consequently, the prevalence of CT infection in the present study cannot be generalized to high-risk populations outside the clinic.

6.2.2 Clinical and Microbiological correlations

Culture positive infections in 18.9% of the cases in the present study suggest active chlamydial replication in these cases. Increased numbers of viable organisms being shed and isolated in culture may indicate heightened potential for transmission in persons with clinical signs of urogenital infection. Acute urethritis and cervicitis are more often seen in a STD clinic compared to other clinical syndromes such as PID, epididymitis and infertility. The present series observed higher isolation rates in urethritis (19%) and cervicitis (20.8%) cases. From a prevention perspective, it is of considerable importance to identify and treat men and women with acute urethritis and cervicitis, as they may be most likely to transmit infection.

The isolation rates were seen higher in the younger age groups. Many studies have found age to be inversely correlated to culture positivity. Acquired immunity to Chlamydia, with increasing age reflecting increased exposure and subsequent resistance to infection could explain this inverse relation ship. Developmental rather than immunologic phenomena could also explain this. In women, changes in the
endocervical epithelium with respect to age may affect the recovery of *C. trachomatis* (Barnes *et al.*, 1990). However in men, such age related changes in the urethra have not been described. The limitation of the present study was small numbers of study subjects in the older age groups to show valid comparison.

It has been suggested that clinical signs of genital chlamydial infection are often mild and non-specific in most cases (Clad *et al.*, 1994). There is also sufficient overlap between the symptoms of chlamydial infections and gonococcal infections. Consequently, chlamydial infections cannot be predicted on the basis of clinical grounds alone. In the present study, discharge and dysuria were the predominant symptoms observed in the total cases as well as in the culture positive cases. Even though these symptoms correlated well to culture positivity or with confirmed infection, failed to show a statistically significant association. The lack of association remained stable even when these two symptoms were analysed separately or together. Thus, it appears that the mere presence of these symptoms alone cannot predict genital infections due to *C. trachomatis*.

We observed a poor correlation between *C. trachomatis* positivity and other genitourinary symptoms in this group of symptomatic patients. This suggests that these symptoms observed might be also due to other genitourinary infections or other STDs (non-chlamydial and non-gonococcal) that were not measured in the study. Finally, *C. trachomatis* positivity could not be attributed to any particular symptom. The findings of this study agree well with that of a study by Tchoudomirova *et al.*, (1998). Some of the earlier studies have shown certain cervical examination findings
in women (cervical ectopy, induced bleeding or friability, mucopurulent/yellow cervical discharge etc.) to be useful in predicting the infections with *C. trachomatis* (Brunham et al., 1984; Nugent and Hillier, 1992). However, we did not observe statistically significant correlation between these cervical signs and *C. trachomatis* infection. Our observations are in agreement with the findings of a recent study from North India (Viswanath et al., 2000) which has shown that the present syndromic algorithms based on speculum assisted clinical evaluation was not helpful in predicting cervical chlamydial infections.

Our results emphasize the need for a universal screening strategy in STD clinic attendees to reduce the rate of CT infections. Clinical management based on syndromic approach may be often ineffective. Firstly, the presence of symptoms and clinical predictions may not always correlate with *C. trachomatis* genital infections. This was evident by the observations in the present study and by others as well. Secondly, it is known that up to 80% of women and 50% of men with *Chlamydia* do not demonstrate symptoms of disease (Black, 1997; Van Der Pol et al., 2001). Thus, in the light of these considerations, it should be speculated that syndromic management alone may not be of help in controlling genital chlamydial infections. Moreover, over treatment by syndromic assessment could also lead to problem of emergence of drug resistance in view of the recently reported drug resistant strains of *C. trachomatis* (Somani et al., 2000).
6.2.3 Analysis of risk factors

The association of different sexual risk variables to *C. trachomatis* infection may differ with respect to study populations and sexual behaviour. STD patients represent a high-risk group compared to the normal population in the community. Therefore, every sexual/behavioural factor may have a role in the acquisition of *C. trachomatis* infection. In the present study, the risk determinant analysis showed significant correlations for sexual variables like premarital sex and sex with multiple partners. Use of oral contraceptives (OCs) was found to be a risk factor for genital infection due to *C. trachomatis* in women.

It was observed that premarital sex was one of the risk factor associated with *C. trachomatis* infection in this group of patients, while extramarital sex was not. Sex with multiple partners was found to be associated with *C. trachomatis* positivity in the present analysis. While significant correlation was observed in those who reported to have 2-5 partners, correlation was not significant in those who had more than 5 sexual partners. Differences that existed between the sexual behaviour of men and women could also explain this variability. Most of the married women (except two, rest of the women were married in the study population), reported husband as the only sexual partner. These observations lead to the assumption that those women who were infected might have contracted the infection mostly through their husbands. The above observations indicate the high-risk behaviour of men. A recent study from North India (Mania-Pramanick et al., 2001) reported a high infection rate of 33.3% in
the partners of the infected women studied. It emerges that like other STDs, sexually transmitted chlamydial infections could also be effectively controlled by partner notification and prompt treatment. Immunity against genital *C. trachomatis* in individuals who had multiple contacts was also suggested (Munk *et al.*, 1999). However, it is reasonable to think that possible repeated exposure to *C. trachomatis* infection by multi-partner sex might only increase the risk of infection rather than immunity.

The use of oral contraceptives (OCs) as a risk factor for *C. trachomatis* has been a matter of debate. It has been suggested that risk of *C. trachomatis* is increased several fold (range from 2.1-2.5 in most studies) in users of OCs (Washington *et al.*, 1985). OC use increases the accessible transitional zone (ectopy), thereby making the cervix more susceptible to infection with *C. trachomatis* (Cottingham and Hunter, 1992). However, another suggested explanation is that the ectopy increases the likelihood of detecting the infection because of effective sampling in OC users (Barnes *et al.*, 1990). Even though, isolation of the organism is mostly dependant on the size of the transitional zone for effective sampling, sensitive molecular methods like PCR are independent of these factors. In the current study, OC use was associated with both culture and PCR positivity and therefore, the detection bias could not have affected this association. However, to address the effect of OC use more accurately, an analysis including the timing of OC use in relation to the occurrence of *C. trachomatis* infection is needed.
It should be emphasized that our findings are based on the infected individuals in a small series of STD patients, and it cannot be excluded that the associations are confounded by behavioural changes over time that we could not adjust for. Further investigations using a large sample size to evaluate the association of the risk factors are ideally needed in a prospective design.

6.3 EVALUATION OF CONVENTIONAL AND MOLECULAR DIAGNOSTIC METHODS FOR *C. TRACHOMATIS*

6.3.1 Isolation in cell culture and antigen Detection by DFA

Isolation was observed in 18.9% of the patients. Antigen detection was slightly sensitive over culture (24.5%). Cell culture systems using shell vial method were reported to have higher sensitivity than microwell plate systems for the isolation of *C. trachomatis* (Yoder et al., 1981; Stamm et al., 1983). In this study, a 24 well microwell plate system was used for chlamydial isolation and this could have reduced the diagnostic sensitivity of culture. DFA detected the presence *C. trachomatis* specific antigen in all the culture positive cases except a case of female endocervicitis. Possible failure of immunochemical reactions by the presence of inhibitory substances (such as antibodies in the secretions) has been suggested (Lin et al., 1992). DFA technique has detected additionally 9 (6.3%) of the positive cases. Taking account of the rapidity and technical simplicity, DFA test could be a useful diagnostic test for genital chlamydial infections. However, there is a subjective element in the
microscopic evaluation and interpretation of results and therefore, care should be taken to maintain the diagnostic accuracy by proper training.

6.3.2 Study on the usefulness of serological markers

Many of the initial studies have favoured the use of serology in the diagnosis of genital chlamydial infections. The evolution of molecular diagnostic tests have changed this concept and emphasized that the value of serology in pinpointing active or ongoing infections is limited. The usefulness of serology can be determined by comparing the humoral responses of patients with proven \textit{C. trachomatis} infection with those of the healthy controls of similar ages and sex ratios (Bas \textit{et al.}, 2001). This is particularly true for populations with high seroprevalence and prevalence of infection e.g., those from a STD clinic. In the present study, the prevalence of anti-\textit{C. trachomatis} antibodies (IgM, IgA and IgG) was significantly high in the patient groups and in the proven cases of chlamydial infection \((P<0.05)\) compared to the healthy controls. The seropositivity in the apparently healthy controls was significantly low \((P<0.05)\) and this suggests a low prevalence of anti-\textit{C. trachomatis} antibodies in the general population.

In the present study, seropositivity with IgM or IgA, but not IgG was significantly correlated with proven \textit{C. trachomatis} infection. The presence of ‘IgM alone’ was seen in 13.6\% of the proven cases which indicates that these may be cases of true primary infection while rest of the infected cases in the study population had repeated or recurrent infection (as evidenced by IgG positivity). It is not known
whether these patients have had symptoms in previous episode(s) of infection to seek clinical care. Thus, it is conceivable that IgM is not a reliable marker for acute infection since it is often not present, presumably because the patient might have had previous chlamydial infection and is manifesting an anamnestic immune response to subsequent infections (Black, 1997). The high background of IgG antibodies in the patients who were negative by the direct tests probably indicates previous infection(s) with *C. trachomatis*.

IgG positivity was relatively high in urethritis cases (67.2%) and PID cases (68.2%) when compared to other clinical groups. It is assumed that many of these cases had past exposure to genital chlamydial infections and thus might be cases of recurrent chlamydial disease. The presence of IgA/IgG antibodies was higher in PID cases. It has been shown that repeated exposures to chlamydial infection increase the risk of PID (Hillis *et al.*, 1997).

Looking for a fourfold rise in antibody titer by doing paired sera analysis is often difficult as it can take up to 1 month or longer for antibody titers to rise, and the delay is not appropriate to the time frame necessary for therapy and management of patients (Black, 1997). Moreover, it is rare to observe such rising titers in uncomplicated lower tract infections. In contrast, an elevated antibody titer in single serum may be diagnostically suggestive in the case of more deep-seated infections such as PID, infertility etc (Taylor-Robinson, 1996). However, caution should be exercised because seropositivity itself is not highly indicative of active infection and high antibody titers do not always correlate with the detection of chlamydiae, being
more often associated with chronic or recurrent disease. The analysis of anti-CT IgG estimation in single serum specimen by the immunoperoxidase test has shown that high titer of IgG antibodies (1:128) were present in female upper genital tract disease groups. It is known that repeated episodes of *C. trachomatis* infection could lead to severe sequelae in women. Previous studies have shown the association of high levels of the IgG antibody with the complications of the female upper genital tract and severity of the disease (Moore *et al.*, 1982; Anestad *et al.*, 1987; Chaim *et al.*, 1989). Thus it appears that single serum IgG titer estimation may be diagnostically helpful to identify deep-seated upper genital tract infections with *C. trachomatis* in the absence of a paired serum specimens.

Our results corroborates with the findings of a study (Theunissen *et al.*, 1994) conducted at a hospital setting in Rotterdam, The Netherlands which demonstrated that *C. trachomatis* infection was detected more often in PID patients using serology in comparison with culture or PCR. In the present series, about 68% (15 out of 22) PID cases that were not detected by culture or PCR were serologically positive by any one of the markers.

The overall seropositivity (by any one of the serological markers) in the study population was 68.5%, which was significant compared to the control group (26%). Moreover it was seen that 81.8% of the proven *C. trachomatis* cases were seropositive by any of the serological marker. The failure of serological detection in 18% of the proven cases could indicate that these cases may have not been seroconverted at the time of their presentation to the clinic. These results suggest that serology may be
useful in identifying most of the active infections only if all the three serological markers (IgM, IgA and IgG) are simultaneously tested.

The present study leads to the conclusion that serology cannot replace the sensitive and specific direct tests, but can assist in the diagnosis of genital chlamydial infections. Reliable serological tests could be used for CT diagnosis if direct tests fail or difficult to perform as in most of the upper genital tract infections. Though the number of cases in these clinical groups in the present series is small, our findings suggest the utility of serology in the diagnosis and management of female upper genital tract infections.

6.3.3  PCR assays for C.trachomatis

Nucleic acid amplification tests have revolutionized the Chlamydia diagnostic research owing to their high sensitivity and specificity over other diagnostic methods. The most widely known of the DNA amplification technologies is polymerase chain reaction (PCR), which has emerged as a powerful tool for the diagnosis of C.trachomatis infection over the years. The performance of the PCR technique may vary with different settings and with specimen types. Some of the initial studies have reported the sensitivity of PCR similar to or less than that of culture or antigen detection assays (Ostergaard et al., 1991; Palmer et al., 1991; Wu et al., 1992). However, these studies have compared PCR with culture alone or in combination with less sensitive methods like enzyme immunoassays. Later studies have shown that PCR is superior to culture and antigen detection methods and has been shown to be

In the present study, the in-house PCR assays were significantly more sensitive than isolation in cell culture (*P*<0.001). By using plasmid PCR, the number of positive patients increased from 27 (18.9%) to 46 (32.2%), thus increasing the diagnostic power. The plasmid PCR and MOMP PCR have shown good concordance for genital specimens. Only two male urethral specimens were not positive by MOMP PCR. These two cases could not be confirmed as samples were insufficient for retesting by either of the assays with same or more amount of starting material. Follow up specimens were not possible from these patients, as they had received treatment on the syndromic basis. Even though scored as false positives for analytical purposes, diagnostically, it should be speculated that the likelihood of *C. trachomatis* infection could not be excluded in these cases of high-risk symptomatic patients.

Overall, PCR assays showed better detection rates in genital specimens compared to urine specimens. Especially in men, genital specimen PCR picked up more number of cases than urine PCR. Although urine PCR testing offers noninvasive testing and facilitates screening in asymptomatic men, it may miss very low copy number infections. It is important to consider that failure to identify such low-level infections in men presumably allows for the maintenance of large untreated reservoir of infection in the population and may transmit the disease to their female partners. In our observations, PCR testing of properly collected genital swab specimens appear to be more efficacious compared to testing of urine specimens in the symptomatic males.
The swabbing process in the infected male urethra induce shedding of more infected cells thereby increasing the sensitivity of *C. trachomatis* DNA detection. The higher sensitivity of urine PCR in some studies (Toye *et al.*, 1996; Young *et al.*, 1998) may reflect the inadequate swab material collected from urethra of asymptomatic males screened. Our results corroborate with those of Weisenfeld *et al.*, (1994) and Crotchfelt *et al.*, (1997) who observed increased PCR sensitivity in genital swab specimens of males. Crotchfelt *et al.*, (1997) showed that when using PCR, sensitivity of urine was 91.1% compared with 99.3% for urethral swabs. The study done by Weisenfeld and colleagues (1994) gave a sensitivity of 98.4% for urethral swab and 87.1% for first void urine.

Crotchfelt *et al.*, (1997) found the sensitivity of cervical swab PCR was 94.1% and the urine PCR 82.4%. Our study has also shown that both plasmid and MOMP PCR assays were more sensitive in cervical swabs than in urine (100% vs. 90.9%). It is generally accepted that a small proportion of women may have localized endocervical or urethral infection without the involvement of other site (Quinn *et al.*, 1996). Thus, it may be assumed that 2.5% of the women (genital PCR positive, urine PCR negative) had *C. trachomatis* infection localized to endocervix only. Three patients (women) who were positive by plasmid urine PCR showed negative results with MOMP urine PCR. However, the endocervical swab specimens of these patients were positive by both the PCR assays. These observations, while indicating low level infection with *C. trachomatis* in these women (these cases were also positive by DFA in cervical swab smear), also support the findings of previous studies which suggested
that plasmid-based PCR assays are more sensitive compared to MOMP-based PCR assays (Mahony et al., 1993; Roosendal et al., 1993; Mahony et al., 1994).

The sensitivities of in-house and the commercial PCR assays may differ due to differences in the procedures of DNA extraction, amplification and detection. In the present study, the in-house plasmid PCR was 9% more sensitive than the commercial Amplicor PCR. This was in accordance with a study done by Shattock et al., (1998). These workers found that the in-house PCR method was more sensitive than the commercial PCR and LCR methods. The reduced sensitivity of the Amplicor PCR in our study was mainly attributed to the PCR inhibition in the urine specimens of 3 culture positive women patients. The advantage of the in-house PCR assays in addition to greater sensitivity was the cost. However the commercial PCR assay is technically less complex, more number of specimens could be handled and offers speedy result.

Presence of inhibitory substances in the samples may give false negative PCR results and could affect PCR sensitivity. In the current study, Amplicor internal control detection kit was used to identify the presence of PCR inhibitors in urine as well as swab specimens. While none of the genital swab specimens showed PCR inhibition, a high rate (6.3%) of inhibition was observed in the urine specimens. PCR inhibitory substances were present in 8.6% of female urine samples whereas it was 3.2% in male urine samples. These percentages were comparable to those reported by others for urine specimens (Mahony et al., 1998; Morre et al., 1999b; Van Der Pol et al., 2001). The phenol chloroform extraction and boiling for 10 minutes removed
inhibitory activity from the urine specimens, facilitating successful amplification with the in-house PCR assays. A DNA spiking method (C.trachomatis L2 DNA corresponding to 1 i.f.u. was spiked on to the aliquots of extracted samples prior to amplification) was used to ensure the removal PCR inhibitory activity in the samples identified by the Amplicor internal control detection.

By using cell culture, antigen detection and PCR assays, the number of confirmed positive cases determined in the present study was 44 (30.8%). Our data revealed that only 82% (36/44) of the truly infected patients gave positive results with both genital specimens and urine specimens, while the remaining 18% were positive by PCR in genital specimens only. We observed less intense or weak PCR bands in the case of the 8 patients (6 men and 2 women) who were positive by genital swab PCR, but negative by urine PCR. Moreover, as per clinical records, these patients reported acquiring illness in less than one month presenting with mild symptoms. These observations indicate that they might have had low-level infection at the time of attending the clinic and might not have sufficient target DNA in their urine samples to be amplified by PCR.

The results of the present study emphasize the diagnostic value of PCR, taking account of increased detection of the truly infected patients, both in men and women. For example, by using the confirmed PCR results, the number of C.trachomatis positive cases increased from 27(18.9%) to 44 (30.8%). Nearly 39% of the infected patients, who were not positive by culture alone, would have been left undiagnosed if PCR testing was not performed. In both men and women, PCR testing increased the
rate of detection compared to culture or antigen detection. In symptomatic patients, PCR sensitivity seems to be high in genital swab specimens. Urine PCR testing is a useful alternative to genital swabs. Taking account of the high rate of PCR inhibition in urine, it needs to be emphasized that the presence of inhibitory substances should be monitored to avoid false negative PCR results.

The multiplex Amplicor PCR kit was used to detect both *C. trachomatis* and *N. gonorrhoeae* simultaneously in the urine specimens. Based on Amplicor test results, 7.7% of the patients were positive for both *C. trachomatis* and *N. gonorrhoeae*. The gonococcus positivity by Amplicor PCR was analysed further in the confirmed cases of *C. trachomatis* infection, which revealed that the co-infection rate was 8.4%. This was comparable with results of Lin et al., (1992) who observed a co-infection rate of 10% among the STD patients screened. More recent studies reported comparatively low rate of co-infections. Xu et al., (1998) and Cecil et al., (2001) observed 5.2% and 4.5% co-infection rates respectively in their studies. This was probably due to the very low positivity observed for *N. gonorrhoeae* in the respective studies. The observation that 27.3% of the proven *C. trachomatis* positive cases had concomitant gonococcal infections in our study was consistent with that reported by Xu et al., (1998). The latter observed that 23.3% of the *Chlamydia* positive cases were having gonococcal infection.

Our observations that *C. trachomatis* was positive in 46.2% of the gonococcus positive cases was similar to the findings of a study done in a STD clinic setting in Jamaica which showed that 48% of the gonococcus patients were infected with
C. trachomatis (Dowe et al., 2000). The high rate of chlamydial co-infection in the gonococcus positive urethritis cases (53.3%) observed in the present study justifies the current policy of National AIDS control organization (NACO) of India advocating dual therapy for a gonococci smear positive urethritis cases as a part of syndromic treatment.

Our results have implications on the diagnosis and treatment of both C. trachomatis and N. gonorrhoeae infections. Even though, treatment of these infections is primarily based on the syndromic judgement, which is adopted in many STD referral centers in India, our observations reinforce the importance of testing for both infections. The convenience of simultaneous testing for both the organisms from one urine sample by the commercial PCR assay is an important consideration. However, cost effectiveness should be worked out.

Many studies have previously compared various commercial assays, culture systems, DFA and in-house PCRs for C. trachomatis (Catry et al., 1995; Smith et al., 1993; Shatlock et al., 1998) although few have evaluated the assays using a common set of specimens. For the diagnosis of C. trachomatis, the test of choice is dependant upon study populations and settings. In a developing country like India, where diagnostic facilities for C. trachomatis are limited, it is important to know the efficiency of different diagnostic methods. Mutiple test evaluations have not been done in India. It is also important to understand the utility of these diagnostic tests in different clinical conditions. In the current study, an array of diagnostic modalities for C. trachomatis, both conventional and molecular markers have been evaluated.
The diagnostic value of the non-culture tests was analysed by taking culture as the reference standard. Even though, this analysis did not affect the estimation of performance values of conventional methods like antigen detection and serology, have drastically affected the estimation of specificities and predictive values of the PCR assays. Antigen detection by DFA has shown good sensitivity, specificity and predictive values when culture was taken as the gold standard. This indicates that almost all the positive cases which are detected by culture could be detected by the DFA test also. Because of the higher sensitivity of amplified assays compared to culture, use of culture results as the only standard increases the sensitivity and decreases the specificity calculated for the NAAT (Van Der Pol et al., 2001). The in-house PCR assays showed 100\% sensitivity (as all the culture positive cases were positive by these PCR tests). The reduced sensitivity of commercial Amplicor PCR was due to PCR inhibition in urine samples of three culture positive cases.

6.3.4 Use of an expanded gold standard for the evaluation of tests.

It is well recognized that the sensitivity of cell culture does not meet the demands of a reference standard for nucleic acid amplification methods. Several recent studies have shown that Chlamydia culture, previously considered as the gold standard has a sensitivity ranging from 50\%-85\% in expert laboratories when compared to the molecular amplification assays (Jaschek et al., 1993; Schachter et al., 1994; Domeika et al., 1994; Quinn et al., 1996).
Many investigators have been adopting a strategy known as discrepant analysis in which an additional confirmatory assay is used to resolve the initial discordant results. However, this method may introduce bias as testing of the discrepant samples alone invariably results in the overestimation of sensitivity and specificity of the tests under evaluation (Hadgu, 1996). The alternative approach is to use a combination of tests to establish an expanded gold standard for the evaluation of diagnostic tests (Goessens et al., 1997; Van Dyck et al., 2001). This implies that the tests used for deriving the expanded gold standard should be done on all the specimens. In the present study, a combination tests including cell culture, DFA, plasmid and MOMP PCR assays (genital swabs) were used to establish an expanded gold standard. A patient was considered truly infected if positive by culture or if positive by any two of the other tests. By applying this strict reference standard, existing differences between different diagnostic methods could be highlighted.

By applying the expanded standard, the culture sensitivity in our study was 61.4%. The sensitivity of urethral culture for men was 50% and that of endocervical culture for females was 72.7%. These percentages are comparable to those observed by others (Goessens et al., 1997; Young et al., 1998) Thus, it is conceived that culture is not an adequate reference standard for evaluating molecular methods. The present analysis has shown that both plasmid and MOMP PCR assays performed in genital swabs have excellent sensitivities and specificities, which place them as the diagnostic tests of choice. The absolute sensitivity with these PCR assays suggests that these methods can detect far more number of infected cases compared to the conventional markers of Chlamydia diagnosis, Among the direct tests, PCR assays improved the
diagnosis of *C. trachomatis* in all the clinical categories. Especially in male urethritis group of patients, PCR detected around 10% of the positive cases in addition to culture and or antigen detection. However, in our study, these molecular assays have shown comparatively lower sensitivity in urine specimens. PCR tests in urine (both in-house and commercial) have shown more or less similar sensitivities. The in-house plasmid PCR was slightly sensitive over the other PCR systems. All the PCR assays in urine samples showed absolute specificity. Our data suggests that PCR testing in urine could be a diagnostic alternative in case of difficulties in obtaining swab specimens from the symptomatic patients. In view of the possible PCR inhibitory activity, it may be necessary to monitor the presence of PCR inhibitory substances in urine samples to avoid false negative results.

Antigen detection by the DFA method appears to be a good diagnostic alternative in resource-limited settings where culture or PCR could not be performed. In the present study, DFA test picked up more cases of urethritis and cervicitis cases than culture. This shows that it could be useful in the early diagnosis of symptomatic chlamydial infections of lower genital tract, which are highly prevalent in settings like STD clinics. The poor sensitivities, specificities and predictive values of serological markers make them unacceptable for the diagnosis of active chlamydial infections. However, they could be helpful in the diagnosis and management of complicated chlamydial infections of the upper genital tract.
6.4 ASSOCIATION BETWEEN CT INFECTION AND HIV INFECTION

6.4.1 HIV status in proven Chlamydia cases

It was observed in the present study that HIV serostatus was significantly correlated to *C. trachomatis* infection. Recent studies suggest that *C. trachomatis* infection of the lower genital tract may facilitate the transmission of HIV (Stamm, 1999). The invasive intracellular pathogenesis of *C. trachomatis* cause substantial damage to the genitalia thereby increasing the risk of infection by HIV. It has been demonstrated that acute *C. trachomatis* infection had an association with HIV infection independent of the sexually transmissible risk factors (Ohshige *et al*., 2000).

The observation that HIV positivity was significantly greater in males compared to females (27% vs. 8.6%) suggests the high-risk sexual behaviour of men in this study population. Our observation revealed that 50% of the HIV positive cases happened to be proven *Chlamydia* positive cases. It is not known whether the exposure to HIV occurred prior to chlamydial infection in these cases. It could be speculated that *C. trachomatis* was prevalent prior to the HIV exposure among the STD cases because the rate of the CT-IgG antibody positive individuals was much higher than that of the HIV seropositive positive individuals (58.7% and 16.8%) and therefore, it is not likely that the lower prevalence of HIV induced a higher prevalence of *C. trachomatis*. 
6.4.2 *Chlamydia* positivity in proven HIV cases

It has been suggested that immunological changes due to HIV infection may favour *C. trachomatis* infection (Miller et al., 1992; Cohen et al., 1995). Immunosuppression due to HIV may lead to more aggressive chlamydial disease conditions like PID in those who are infected with *C. trachomatis* (Thomas and Simms, 2002). Brunham and colleagues (1996) have recently shown that the risk of chlamydial infection was higher in those having HIV infection than those without. Women with HIV infection had both an increased prevalence of chlamydial infection and an increased rate of reinfection. We also observed significantly higher *C. trachomatis* positivity in HIV seropositive women compared to the HIV seronegative group. This raises interest for further prospective studies with more number of subjects in both arms, which would be helpful in understanding the incidence, risk, recurrence rates and transmission patterns of *C. trachomatis* infection vis-a-vis HIV infection status.