

***Chlamydia trachomatis* and human papillomavirus infection in Indian women with sexually transmitted diseases and cervical precancerous and cancerous lesions**

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Objectives Sexually transmitted diseases (STDs) and anogenital cancers are the major health problems in Indian women but no reliable estimate of the prevalence of either genital chlamydial infection or human papillomavirus (HPV) infection in STD patients is available. The aim of this study was to detect the frequency of *Chlamydia trachomatis* and the most prevalent high-risk HPV type 16 (HPV 16) infection in Indian women, with STDs and precancerous and cancerous lesions of the uterine cervix by polymerase chain reaction (PCR), and their comparison with those of conventional serology and antigen tests used for *C. trachomatis* detection.

Methods Endocervical swabs or scrapes were collected from 50 women with STDs and 30 normal healthy women attending the STD clinics of Smt. Sucheta Kripalani Hospital, New Delhi. Scrapped cervical cell specimens were also collected from 50 women with precancerous and cancerous lesions of the uterine cervix. Detection of *C. trachomatis* and HPV was carried out by PCR using chlamydia and HPV genome-specific oligonucleotide primers. The detection of chlamydial antigen and IgG-specific antibodies was carried out by enzyme immunoassay (EIA) and serological enzyme-linked immunosorbent assay (ELISA), respectively.

Results A chlamydia plasmid-based PCR assay detected 50% (25 of 50) positivity of *C. trachomatis* in STD patients and HPV 16 DNA was found in 30% (15 of 50) of these cases which are significantly higher than those found in healthy controls. The PCR estimate of chlamydia was found to be higher than its reported frequency by tissue culture. The EIA could detect chlamydial antigen in only 13 cases (26%) while serological ELISA revealed evidence of chlamydial IgG-specific antibodies in 26 (52%) cases. Interestingly, in women with precancerous and cancerous lesions, the rate of HPV 16 infection was very high (52% and 72%, respectively), whereas the frequency of chlamydia infection was found to be 12–22% only. Occurrence of other sexually transmitted agents was also evaluated in the women.

Conclusions This is the first PCR estimate of genital chlamydial (50%) and HPV 16 (30%) infection in STD patients and women with precancerous and cancerous lesions of the uterine cervix in India. The PCR method seems to be a good alternative to tissue culture.

Keywords *Chlamydia trachomatis*, human papillomavirus (HPV), sexually transmitted diseases (STD), cervical precancer, cervical cancer, polymerase chain reaction (PCR), serology

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INTRODUCTION

Chlamydia trachomatis, an obligate intracellular Gram negative bacterium, is one of the most common sexually transmitted

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agents which causes a wide spectrum of diseases including uterine cervical lesions [1,2] in humans. It also causes ocular disorders, such as trachoma and conjunctivitis. Infants born to pregnant mother with genital chlamydial infection are also often at risk of developing conjunctivitis and pneumonia. *Chlamydia trachomatis* infection may lead to serious sequelae such as endometritis and salpingitis which often cause infertility in women [3,4]. The serotypes D to K of *C. trachomatis* are mainly associated with sexually transmitted diseases (STDs). Epidemiological studies have revealed a prevalence of the bacteria in

about 5% of normal population at large although this frequency is reported to vary between 20 and 40% in STD patients [5–7]. However, chlamydial infections are often asymptomatic which could contribute to further spread of the disease. Therefore a sensitive, rapid and reliable method for early diagnosis of *C. trachomatis* infection is important for treatment with antibiotics.

In India, sexually transmitted diseases and anogenital cancers are the major health problems in women. Annually about 100 000 women develop cervical cancer and approximately, 40–50% are infected with one or the other sexually transmitted agents [8]. A high prevalence of human papillomaviruses (HPVs) and their role in the development of cancer of the uterine cervix have been well-documented in Indian women [9,10] but no reliable estimate of the prevalence of genital chlamydial infection is available. Furthermore, no information is available on the prevalence of HPV in STD patients in India. Using conventional serological and antigen detection assays, a prevalence rate of 30 to as high as 81% has been reported for chlamydia in asymptomatic women [11–13]. There is, so far, only one report of cell culture from India which showed the prevalence of chlamydial infection to be about 40% in STD patients [14].

Since all these conventional diagnostic serology or immunoassays, including the cell culture methods, are associated with one or other limitation [15,16] and no polymerase chain reaction (PCR) assay has been employed, we have used a plasmid-based PCR for the detection of *C. trachomatis* in endocervical smear/scrape specimens of women with STD as well as pre-cancerous and cancerous lesions of the uterine cervix. In addition, diagnosis was carried out for the 'high-risk' HPV type 16 (HPV 16) which is almost exclusively prevalent in the Indian population and is considered to be a major etiologic agent for the development of cervical cancers [8,17] in women.

MATERIALS AND METHODS

Study groups

Endocervical swabs or scrapes were collected from 50 symptomatic women aged between 15 and 44 years (mean age 27.2 ± 9 years) who were attending the STD clinics of Smt. Sucheta Kripalani Hospital, New Delhi. Thirty normal healthy women of similar age group (28 ± 8.5 years) with no previous or present history of promiscuity, STD or other infectious diseases attending hospital out-patient-department for either birth control information or other health check-ups were included as controls. In addition, 50 women (mean age, 30 ± 9.6 years) with precancerous (mild or moderate dysplasia) and 50 (mean age, 33.2 ± 8 years) with cancerous lesions of the uterine cervix were recruited from Lok Nayak hospital, New Delhi and scraped cells were collected. All the women

were subjected to thorough clinical examination and a case history was recorded. All information regarding similar age group, marital status, socio-economic condition, occupation, parity, age at marriage, obstetric history, history of any sexually transmitted diseases in herself or in her husband and the method of contraception used, etc. were collected. Those who had received antibiotics in the last 4 weeks were excluded from the study.

Specimen collection

The ectocervix was first cleaned with a cotton swab to remove excess mucus and exudate. Three endocervical swabs were obtained for (i) microscopic examination of pus cells (ii) for culture of other pathogens, namely gonorrhoea, genital herpes virus (HSV2), candidiasis, bacterial vaginosis, etc. and (iii) for antigen detection of *C. trachomatis*. Endocervical cell scrapings were obtained by rotating the Ayer's spatula at 180° in the endocervical canal and transferred to 15 mL plastic vials containing 5 mL of sterile phosphate buffered saline (PBS) and stored at -70°C in a deep freezer until isolation of DNA for PCR assay. In addition, 5 mL of peripheral venous blood was collected from each patient for chlamydial serology, ELISA for HIV and VDRL (Veneral Disease Research Laboratory) test.

Serological tests

Serological tests were carried out for the detection of IgG specific antibodies to *C. trachomatis* antigen in patients' sera by enzyme immunoassay [18] (*Chlamydia* IgG; Melotest, Barcelona, Spain). The assay utilizes an inactivated specific antigen coated on to microtiter wells, which binds to the specific antibodies present in the samples. An antihuman IgG antibody contained in the antibody-enzyme (horse radish-peroxidase) conjugate solution, binds to the antibodies in the test sample and finally the color development was measured at 450 nm using a spectrophotometer. The samples with concentration less than 9 A.I.U./mL was considered antibody negative, 9–11 A.I.U./mL as equivocal and more than 11 A.I.U./mL was taken as antibody positive.

Antigen assay

Detection of *C. trachomatis* antigen in endocervical specimens was carried out by ELISA using a *Chlamydia* ELISA kit (CELISA) [19–21] (Cellabs Pty. Ltd, Brookvale, NSW, Australia), which detects chlamydial lipopolysaccharide (LPS) that binds to the microtitration strips. Monoclonal antibodies added in the subsequent step bind to the LPS, if present. A peroxidase conjugated antimouse antibody was added in the third step binds to any antigen-antibody complexes in the microwells.

The substrate used was 3,3,5,5' tetramethyl benzidine (TMB) which produces a blue-colored product in the positive wells. The absorbances were read at 450 nm using a microwell plate in a spectrophotometer. For each run, one positive and two negative controls were included.

DNA extraction

DNA was extracted by a nonorganic method as described previously by us [22]. Briefly, the scraped cervical cells were washed once in 1 mL of cold PBS and twice in chilled Tris-Triton buffer (TTB) containing 10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 300 mM Sucrose and 0.8% Triton X-100. The cell pellet was again washed in cold Tris-EDTA buffer (TEB) containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 10 mM NaCl. Finally, the cell pellet was resuspended in 200 μ L TE buffer supplemented with 1.25 mg/mL proteinase-K (Boehringer Mannheim, Germany) and incubated at 65 °C water bath for 2 h. This DNA solution was directly used for PCR amplification.

Polymerase chain reaction

The PCR was carried out for amplification of a 201 bp sequence of the cryptic chlamydia plasmid extending from 4761 to 4961 bp (EMBL Gene Bank accession number 4133141) using the following primers: CC3-5'-TAG TAA CTG CCA CTT CAT CA-3' and CC4-5'-TTC CCC TTG TAA TTC GTT GC-3'.

The primer sequences were synthesized by an AB1 DNA synthesizer (Model 381 A; Applied Biosystems Inc, Foster City, CA, USA) and PCR amplification was carried out in a DNA thermal cycler (Perkin Elmer Cetus, Roche, NJ, USA) using *Taq* DNA polymerase (AmpliTaq: Cetus Corporation, Foster City, CA, USA). The amplifications were carried out in a 50 μ L reaction mix containing 1 μ g sample DNA, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl₂, 16.7 mM ammonium sulphate, 6.0 mM EDTA (pH 8.0), 2.5 mM dNTPs (dATP, dCTP, dTTP, dGTP), 1 μ M each of *Chlamydia* primers CC3 and CC4 and 0.5 units of *Taq* DNA polymerase (Cetus). The reaction mixture was overlaid with 50 μ L mineral oil to prevent evaporation. The PCR amplifications were performed for 35 cycles, each with 30 s of denaturation at 94 °C, annealing at 55 °C and extension at 72 °C except the first cycle when denaturation was done for 5 min. In the last cycle extension at 72 °C was prolonged for 5 min. Detection of HPV 16 DNA sequences was carried out using primers from its conserved upstream regulatory region (URR). The HPV 16 primers which gave amplifier of 247 bp were as follows: URR-1 5'-AAG GCC AAC TAA ATG TCA C-3' and URR-2 5'-CTG CTT TTA TAC TAA CCG G-3'.

The PCR amplification procedures were followed as described elsewhere [10,23]. The quality of DNA was checked by simultaneous amplification of the β -globin gene in all the

samples. The β -globin primer sequences were: β -globin-1 5' GAA GAG CCA AGG ACA GGT AC 3' and β -globin-2 5' CAA CTT CAT CCA CGT TAC ACC-3'.

The amplimer size was 268 bp. The desired positive signals visualized as PCR amplimers of 201 bp (Figure 1a) and 217 bp (Figure 1b) for *C. trachomatis* and HPV 16, respectively, in a 3% Nusieve-agarose gel (FMC BioProducts, Rockland, ME, USA) were reconfirmed following their transfer and hybridization with specific DNA probes in Southern blot hybridization.

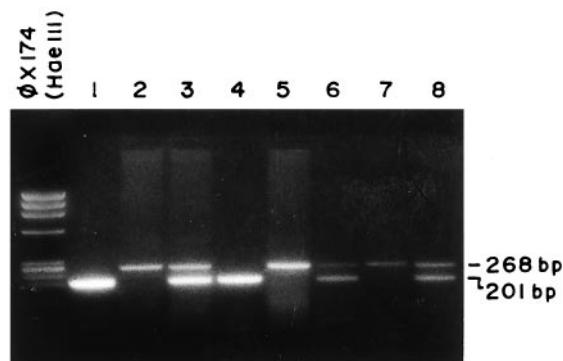


Figure 1a Detection of *Chlamydia trachomatis* plasmid-specific amplification by polymerase chain reaction. Lane 1 is positive control for *C. trachomatis* and lanes 2–8 are the cases. Lanes 3, 4, 6 and 8 show amplification of a *C. trachomatis* specific 201 bp product along with 268 bp amplimer for the β -globin gene as internal control. Lanes 2, 5 and 7 are negative for chlamydia showing amplification of only β -globin gene but absence of chlamydia-specific amplimer. The extreme left lane is *Hae* III-digested *ox174* DNA size marker.

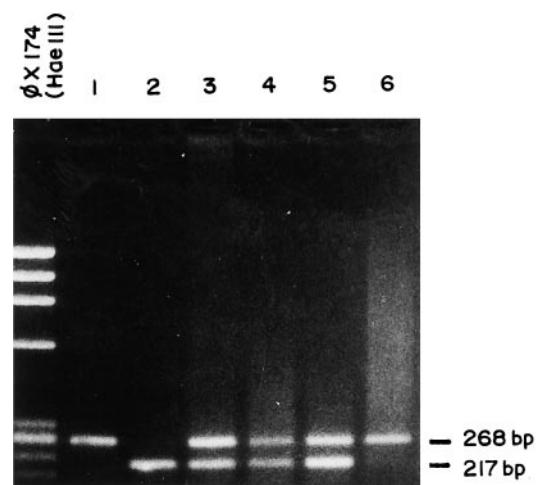


Figure 1b Polymerase chain reaction amplification of human papillomavirus type 16 (HPV 16) DNA along with β -globin gene sequences in endocervical scrapes of patients with sexually transmitted diseases. First lane is *Hae* III digested *ox174* DNA size marker. Lane 1 is placental DNA showing β -globin gene (268 bp) amplification. Lane 2 is CacoX DNA showing HPV 16 gene (217) amplification. Lane 3–5 are endocervical scrapes showing positivity of HPV 16 and β -globin gene while lane 6 is negative for HPV 16.

RESULTS

The results of the PCR analysis of *C. trachomatis* and HPV 16 in 50 cases of symptomatic women with STD along with 30 asymptomatic healthy normal controls are presented in Table 1. The patients were in the age group of 15–44 years, the mean age being 27.2 ± 9 years and the majority (32 of 50, 64%) were in the reproductively active age group of 25–34 years. Forty-five patients (90%) were married and interestingly, only five (10%) had a history of pre- or extramarital contacts and 46 (92%) women had a single partner. Further elicitation of information revealed that a history of concomitant sexually transmitted diseases present in 16 (32%) women and a history of promiscuity was revealed in 21 (42%) sexual partners.

Of 50 cases of STD studied, 25 (50%) were positive for *Chlamydia* whereas only 15 (30%) patients showed presence of high-risk HPV 16 DNA sequences as revealed by chlamydia and HPV 16 genome-specific PCR products in ethidium bromide stained gel (Figure 1a, b). The frequencies were highly significant ($P < 0.001$) when compared with those of the controls (Table 1). Enzyme immunoassay for chlamydia antigen alone could detect evidence of chlamydia positivity only in 13 (26%) cases of which 11 (22%) were found to be positive by PCR. An additional 14 (28%) antigen-negative cases were also PCR positive. Only two (4%) cases were antigen positive but PCR negative. The control women showed 3% positivity for chlamydia by PCR. Specific IgG antibodies against *C. trachomatis* in serological ELISA was detected in 26 (52%) women of whom only 16 were positive by PCR. Interestingly, the remaining nine PCR-positive women (the total being 25) were derived from the 24 antibody-negative patients, whereas 10 patients who were positive for antibody test were negative by PCR.

Analysis of *C. trachomatis* as well as HPV in cervical precancer and cancer cases revealed a low frequency of *C. trachomatis* in both cancer (22%) and precancerous (12%) lesions whereas the frequency of HPV16 was found to be 52 and 72%, respectively. The difference was found to be highly significant ($P < 0.001$). Among the 50 cases of endocervicitis patients analyzed, 39 (78%) patients concomitantly had sexually transmitted diseases such as syphilis, gonorrhoea, herpes progenitalis, bacterial vaginosis, etc., and 11 patients had no history of STDs. Among the chlamydia-infected patients, the majority of them (12 of 25,

48%) also had syphilis besides other concomitant sexually transmitted infections. Interestingly, five of 11 with no history of STD were also found to have chlamydial infections.

DISCUSSION

Polymerase chain reaction is considered to be the most sensitive and specific diagnostic method employed for detecting infectious agents including *C. trachomatis* [24–28]. In India, only a few studies have taken place and this is the first report of a *C. trachomatis* PCR assay showing the frequency of genital chlamydial infection to be 50% in Indian women with STDs. In contrast, a single study which used a tissue-culture method revealed 41% positivity for chlamydia [13]. Although the standard for detection of *C. trachomatis* has been the cell culture [29,30], several clinical and technical factors can lead to false negative results in the culture system [31]. Much care and precision are therefore required for collection, transport, storage and processing of cervical specimens for tissue culture. Furthermore, the culture method is time-consuming, expensive and inapplicable for screening large number of samples at a time. In contrast, several reports [26,28,32,33] including the present data indicate that PCR could serve as a good alternative to tissue culture.

Antigen assay, which is commonly used for *C. trachomatis* detection, appears to be an easier method but it is considerably less sensitive than cell culture. This is evident from the present finding that *C. trachomatis* antigen could be detected in only 26% (13 of 50) of cases and of these only 11 (22%) could be confirmed by PCR. It is possible that the antigen test may not recognize all chlamydia serovars and hence the results obtained by this test may not be reliable. Serological detection of IgG antibody to *C. trachomatis* is also limited by interspecies cross-reactivity leading to a substantial number of false positive or negative results. There is also a high background prevalence of antibodies to *C. trachomatis*. Therefore the diagnostic value of a single high titre of antichlamydial antibodies of IgG class or any other stable high titre is uncertain. A majority of antibody-positive or -negative cases could not be reconfirmed by PCR. This may be because of either past infection or the antibody titre was decreased to a level undetectable by ELISA.

Table 1 Detection of *Chlamydia trachomatis* and human papillomavirus type 16 (HPV 16) DNA sequences in women with sexually transmitted diseases, by polymerase chain reaction

Subjects	Micro-organism	No. (%) positive	Chl + HPV+	Chl-HPV+	Chl-HPV+	Chl-HPV-
STD's (n=50)	<i>C. trachomatis</i>	25 (50)	7 (14)	18 (36)	8 (16)	19 (38)
	HPV 16	15 (30)	–	–	–	–
Controls (n=30)	<i>C. trachomatis</i>	1 (3.3)	–	–	–	–
	HPV 16	4 (13.3)	–	–	–	–

It is interesting to note that majority of chlamydia-infected patients also had syphilis (12 of 25, 48%) besides other concomitant sexually transmitted infections such as gonorrhea, genital herpes simplex virus type-2, condyloma acuminata, candidiasis, bacterial vaginosis, etc. It has been mostly observed that the sexual transmission of HIV infections are generally higher in STD patients [34–37] but serologically it is interesting that no HIV-positive case could be detected in the present study group of STD patients in spite of the fact that HIV infection shows an increasing trend in India [38].

Chlamydia infections may be associated with inflammatory changes in the cervical epithelium, particularly friability of the cervix which has been observed in 16 (45.7%) cases. The prevalence of high-risk HPV 16 is found to be 30% among women with STDs. This is to our knowledge the first report of the frequency of HPV in STD patients from India. This observation is very important since HPV 16 is the type almost exclusively (~90%) prevalent in India [10] and it has been observed that asymptomatic women who harbor high-risk HPV 16 are shown to have a high rate of progression to malignancy [39; Das et al. unpublished]. Such a test, allows early identification of a population at high risk of developing cervical cancer.

It would also be very interesting to see if there is any synergistic effect when both chlamydia and HPV are present in comparison with infection of HPV alone during development of cervical cancer. Observation of a slightly higher rate of chlamydia infection in cancer cases when compared with that of controls or precancers indicates that *C. trachomatis* may play a role as a cofactor with regard to the pathological aggressiveness of the disease.

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