Genital mycoplasma & *Chlamydia trachomatis* infections in treatment naïve HIV-1 infected adults

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Background & objectives: Sexually transmitted infections (STIs) enhance the transmission of human immunodeficiency virus (HIV). Thus, screening for STIs is a routine component of primary HIV care. There are limited data for selective screening guidelines for genital mycoplasmas and *Chlamydia trachomatis* in HIV-infected adults. The aim of the present study was to determine the frequency of genital infections with *Ureaplasma* spp., *Mycoplasma hominis*, *M. genitalium* and *C. trachomatis* in treatment naïve asymptomatic HIV-1 - infected adults and study their association with CD4+ T-cell count.

Methods: First-void urine samples were collected from 100 treatment-naïve HIV-1-infected adults and 50 healthy volunteers. *C. trachomatis* and *M. genitalium* were detected by polymerase chain reaction (PCR). *Ureaplasma* spp. and *M. hominis* were detected by both culture and PCR. Circulating CD4+ cell counts of HIV-1-infected patients were determined from peripheral blood by flow-cytometry.

Results: *C. trachomatis* was detected in 7 per cent of HIV-1-infected adults compared to none in control population. *Ureaplasma* spp. and *M. hominis* showed infection rates of 6 and 1 per cent in the HIV group and 2 and 0 per cent in the control group, respectively. None of the individuals from the patient and control groups was tested positive for *M. genitalium*. A significant association was found between CD4 cell count and detection of *C. trachomatis* in HIV-infected adults ($P = 0.01$).

Interpretation & conclusions: Screening of HIV-infected individuals for *C. trachomatis* infection could be recommended as a routine component of HIV care. The role of mycoplasmas as co-pathogens of the genitourinary tract in HIV-1 infected patients seems to be unlikely. Further longitudinal studies need to be done to confirm these findings.

Key words CD4 cell counts - *Chlamydia trachomatis* - genital mycoplasmas - HIV

Approximately 2.27 million people were estimated to be living with human immunodeficiency virus (HIV) in India in 2008 with an adult HIV prevalence of 0.29 per cent. Sexually transmitted infections (STIs) enhance the transmission of HIV. Hence, a provision of STI services is aimed at preventing HIV transmission under National AIDS Control Programme[1]. Certain genital mycoplasmas like *Mycoplasma genitalium*, *M. fermentans*, *M. penetrans* and *M. pirum* are candidate "co-factors" in the pathogenesis of acquired immune deficiency syndrome (AIDS) [2-4]. In other words, these mycoplasmas act in synergy with HIV to exacerbate the retroviral disease. In HIV-infected

patients, an increased frequency of *Ureaplasma* spp. and *M. hominis* infection rates have also been reported[5,6]. However, published data lack indisputable conclusion and doubts still exist on the role of these organisms in HIV-infection. *Chlamydia trachomatis* is associated with increased genital HIV shedding that may increase HIV transmissibility[7]. In addition, in HIV-infected individuals, infections with *C. trachomatis* and genital mycoplasmas are important biologic markers of behaviour that may expose others to HIV.

Although screening for STIs is a routine component of primary HIV care, there are limited data for selective screening guidelines for genital mycoplasmas and *C. trachomatis* infections in HIV-infected adults. Identification of these infections in HIV-positive adults can help formulate screening strategies and target prevention intervention promoting safer sexual practices. Treatment of these STIs may impact heterosexual HIV transmission.

The hallmark of HIV disease is a profound immunodeficiency resulting primarily from a progressive decline in the number of CD4+ T cells. Although a few international studies have determined the influence of CD4 cell count on genital mycoplasma and *C. trachomatis* infection in HIV infected individuals,[8,9] no study from India is available.

We investigated the *C. trachomatis* and genital mycoplasmas *viz. M. genitalium*, *Ureaplasma* spp. and *M. hominis* infections in treatment naïve asymptomatic HIV-infected adults by conventional and molecular methods. Association between CD4+ T-cell counts and detection of these organisms was also determined.

Material & Methods

Study population: The present cross-sectional study was conducted at the All India Institute of Medical Sciences (AIIMS), New Delhi, from January 2009 to December 2009. Treatment-naïve HIV-1 infected adults attending the Reference HIV- Laboratory in the Department of Microbiology for CD4-cell count were identified by review of patient information chart and laboratory records. One hundred eligible patients (64 males and 36 females) were enrolled after obtaining written informed consent. Pregnant women, children, adolescents and HIV-infected adults with symptoms of genital tract infections were excluded from the study. The control group consisted of 50 HIV-negative healthy volunteers (35 males and 15 females) recruited from the hospital staff.

The HIV serostatus of the study population was determined and confirmed as per National AIDS Control Organisation (NACO 2007) guidelines[10]. Comprehensive demographic, clinical, laboratory and pharmaceutical data were obtained. Both the HIV-1 infected and control groups consisted of sexually active adults who had no history of homosexual behaviour.

The study protocol was approved by the Ethical Committee of the Institute.

Sample collection and processing: Thirty millilitre (ml) of first-void urine (FVU) and three ml of peripheral blood samples in EDTA vials were collected from all participants and processed on the day of collection. FVU samples were collected from all participants and processed on the day of collection. Samples were concentrated 10-folds by centrifugation at 500 *g* for 30 min at 4ºC (Cold Centrifuge, Hermle, Germany) and filtered through 0.45 μm membrane filter (Millipore, USA). The processed samples were subjected to semi-quantitative culture of *Ureaplasma* spp. and *M. hominis*. Aliquots were also tested for *M. genitalium*, *Ureaplasma* spp., *M. hominis* and *C. trachomatis* by polymerase chain reaction (PCR).

Culture of genital mycoplasmas: Two-hundred microlitre of processed urine was inoculated each into 1.8 ml of Pleuropneumonia-like organism (PPLO) broth containing urea and PPLO broth containing arginine for isolation of *Ureaplasma* spp. and *M. hominis*, respectively. Serial 10-fold dilutions starting from 1:10 to 1: 10^5 were prepared. The broths were incubated at 37°C under 5 per cent CO_2 and were inspected twice daily. The broths were incubated for a period of 14 days before discarding them as negative. The broths were subcultured onto PPLO agar plates containing urea for *Ureaplasma* spp. and arginine for *M. hominis*, when a colour change occurred in absence of turbidity indicating a rise in *p*H. The highest dilution which changed the colour of the indicator present in the broth represented the number of the organisms in the sample in colour changing units per ml (CCU/ml). Relevant concentrations were considered >10^4 CCU/ml for *U. urealyticum* and >10^3 CCU/ml for *M. hominis*. *Ureaplasma* spp. and *M. hominis* were identified by their characteristic "fried-egg" colonies on PPLO agar plates and after Dienes' staining. *U. urealyticum* NCTC 10177 and *M. hominis* NCTC 10111 were used as reference strains (National Collection of Type Cultures, London).

Antibiotic susceptibilty test for Ureaplasma spp.: The isolates of *Ureaplasma* spp. were subjected to antibiotic susceptibility testing against azithromycin, doxycycline, ofloxacin and josamycin by microbroth dilution method (Fig. 1). Minimum inhibitory concentrations (MICs) were determined based on the lowest concentration of antibiotic causing a colour change which remained stable after 48 h of incubation. Cut-off MICs for susceptibility, intermediate and resistance for josamycin, azithromycin, ofloxacin were taken as ≤ 2, 4 and ≥8 μg/ml, respectively and for doxycycline the values were ≤ 4, 8 and ≥16 μg/ml respectively[11].

Nucleic acid extraction: DNA was extracted from processed urine samples with QIAamp Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C for PCR testing.

Fig. 1. Microbroth dilution antibiotic susceptibility testing of *Ureaplasma* spp. Row 1: Negative control (Uninoculated broth without drug); Row 2: Positive control (Broth inoculated with isolate without drug); Row 3: Drug control (Uninoculated broth with highest concentration of drug); Row 4-12: Inoculated broth with drug dilutions ranging from 128 to 0.5 μg/ml. [Column A-Doxycycline, C- Azithromycin, E- Josamycin and G - Ofloxacin].

PCR for M. genitalium: *M. genitalium* was detected by PCR targeting the 140 kDa adhesion gene using primers MgPa-1 and MgPa-3 following the protocol described by Jensen *et al*[12]. Briefly, 25 μl amplification reaction mixture contained 2.5 μl of 10× PCR buffer [1× PCR buffer is 10 mmol/l Tris-HCl (*p*H 8.8 at 25°C), 50mmol/l KCl, and 0.1% Triton X-100], 4.5 mM MgCl$_2$, 1 U of Taq polymerase (GeneiTaq, Bangalore Genei, India), 250 μmol/l (each) deoxynucleoside triphosphate mixture, 20 pmol of each primer, 5 μl of sample DNA and ultrapure sterile water. The PCR conditions used were initial denaturation at 95°C for 1 min, cyclic denaturation at 95°C for 1 min, annealing at 67°C, and extension at 72°C for 1 min for 35 cycles, and final extension at 72°C for 6 min in a thermocycler (MJ Research, Waltham, MA) (Fig. 2). The template of *M. genitalium* DNA used as positive control was gifted by Dr M. Jurstrand, Clinical Research Centre, University Hospital, SE-70185, Orebro, Sweden.

Multiplex PCR for Ureaplasma spp. *and M. hominis*: Multiplex PCR targeting the urease gene of *Ureaplasma* spp. and 16S rDNA of *M. hominis* was used to detect the presence of DNA of these two organisms using the protocol by Stellrecht *et al*[13]. Briefly, the 50 μl amplification reaction mixture contained 5.00 μl of 10× PCR buffer [1× PCR buffer is 10 mmol/l Tris-HCl (*p*H 8.8 at 25°C), 50 mmol/l KCl, and 0.1% Triton X-100], 3.0 mM MgCl$_2$, 1.25 U of Taq polymerase (GeneiTaq, Bangalore Genei, India), 400 μmol/l (each) deoxynucleoside triphosphate mixture, 25 pmol
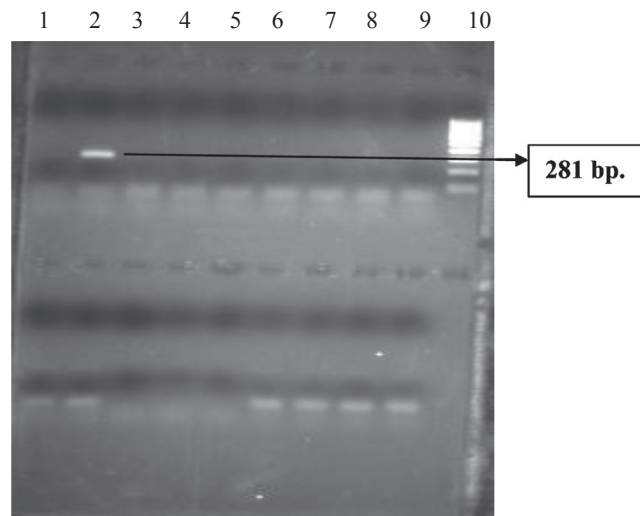

Fig. 2. Agarose gel (1.5%) electrophoresis for detection of *Mycoplasma genitalium* by PCR. Lane 1: Negative control; Lane 2: Positive control; Lane 3-9: Clinical samples- negative; Lane 10: 100 bp DNA Ladder.

of each primer, 16 μl of sample DNA and ultrapure sterile water. The PCR conditions used were 1 cycle of initial denaturation at 95°C for 10 min, followed by 35, two-step cycles of 95°C for 15 sec, 60°C for 1 min, and followed by 5 min at 72°C in a thermocycler (MJ Research, Waltham, MA). Extracted DNAs from *U. urealyticum* -NCTC 10177 and *M. hominis* NCTC 10111 reference strains were used as positive controls. All the isolates of ureaplasmas were further biotyped by a second PCR targeting the multiple banded antigen gene[14].

PCR for C. trachomatis: *C. trachomatis* DNA was detected by PCR targeting a sequence of the cryptic plasmid using primers KL-1 and KL-2[15]. Template DNA of *C. trachomatis* serovar D (ATCC VR-885) was used as positive control in each PCR reaction. The reaction conditions were 2.5 μl of 10× PCR buffer [1× PCR buffer is 10 mmol/l Tris-HCl (*p*H 8.8 at 25 °C), 1.5 mmol/l $MgCl_2$ 50 mmol/l KCl, and 0.1% Triton X-100], 200 μmol/l (each) deoxynucleoside triphosphate mixture, 10 pmol of each primer, 2 U of Taq DNA Polymerase (GeneiTaq, Bangalore Genei, India), 5 μl of sample DNA and ultrapure sterile water in a total volume of 20 μl. Amplification was performed in a thermocycler (MJ Research, Waltham, MA) running 35 cycles, each consisting of a 94°C/1 min denaturation, 55°C/1 min annealing and extension at 72°C for 2 min (Fig. 3).

Samples positive for *C. trachomatis* by cryptic plasmid PCR were confirmed by a second PCR targeting
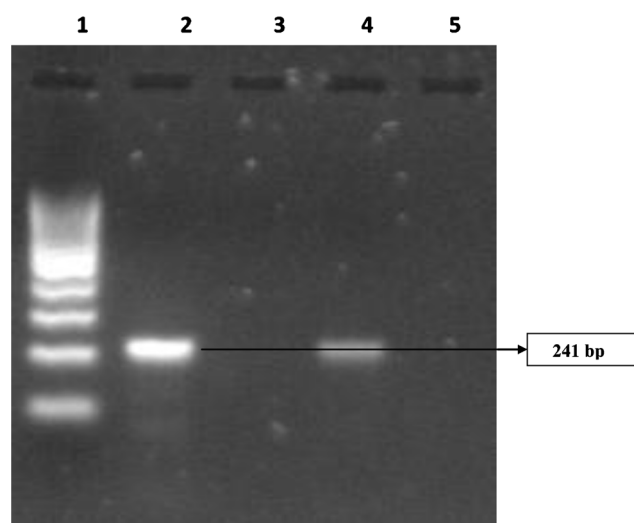

Fig. 3. Agarose gel (1.5%) electrophoresis for detection of *Chlamydia trachomatis* by PCR. Lane 1: 100 bp DNA ladder; Lane 2: Positive control; Lane 3: Negative control; Lane 4: Clinical sample-positive; Lane 5: Clinical sample - negative.

the *omp1* gene as described by Gao *et al*[16]. For *omp*1 gene amplification, 10μl of extracted DNA was added to a reaction tube with the PCR mixture containing 25 pmol (each) of primers NLO and NRO, 200 μmol/l (each) deoxynucleoside triphosphate mixture, 1X PCR buffer (20mM Tris-HCl, *p*H 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$ and 2 U of Taq DNA polymerase (GeneiTaq, Bangalore Genei, India). Amplification was performed with 6 min of initial denaturation at 95°C, followed by 49 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 3 min and chain elongation at 72°C for 3 min. The primers with their sequences and target genes used in the different PCR assays are shown in the Table.

Determination of CD4+ T cell count: Peripheral blood samples (3 ml) of the HIV-infected subjects were collected for CD4+ T- cell count by flow-cytometry (Becton Dickinson FACS Counter) as per standard protocol. Association between CD4 cell counts and detection of the above organisms was evaluated.

Data analysis: Statistical analysis was performed using Fisher's exact test, Chi-square test and Student-t test. *P*<0.05 was considered significant.

Results

The age range of the HIV-1 positive adults was 18 to 45 yr (mean ± SD, 29.970 ± 7.289 yr), and for the HIV negative control group was 18 to 43 yr (29.720 ± 5.272 yr). Majority of the patient population (85%) were diagnosed as HIV-1 seropositive within one year prior to enrolment. Nineteen per cent of HIV-1 infected individuals had CD4+ T cell count of < 200/μl and had AIDS according to the 1993 revised classification system for HIV infection of the Centre for Disease Control and Prevention[17]. The remaining 81 per cent of HIV-1 positive cases with CD4+ T cell count of ≥ 200/μl were asymptomatic.

Seven out of 100 (7%) HIV-1 infected adults were tested positive for genital mycoplasmas compared to one out of fifty (2%) HIV negative healthy controls (*P*=0.20). Among HIV-1 positive individuals, genital mycoplasmas were detected in the samples of 15.8 per cent (3/19) patients with CD4 cell count of < 200/μl (AIDS group) compared to 4.9 per cent (4/81) of cases with CD4 cell count of ≥ 200/μl (non-AIDS group) (*P*=0.09). Thus, a CD4 count of less than 200/μl, was not associated with increased risk of genital mycoplasma infection in HIV positive patients. [Odds Ratio (OR) = 3.61, 95% Confidence Interval (CI) : 0.47- 23.28] In addition, the difference in mean CD4 lymphocyte count between HIV-infected with and without genital mycoplasmas

Table. Primers used in different PCR-based detection assays

Organism	Primer	Sequence	Amplicon size	Target gene	Ref No.
M.genitalium	MgPa-1	5' AGTTGTGAAACCTTAACCCCTTGG 3'	281 bp	Adhesin	12
	MgPa-3	5' CCGTTGAGGGGTTTTCCATTTTTGC 3'			
Ureaplasma spp.*	U4	5'ACGACGTCCATAAGCAACT3'	429 bp	Urease	13
	U5	5'CAATCTGCTCGTGAAGTAATTAC3'			
*M.hominis**	RNAH1	5'CAATGGCTAATGCCGGATACGC 3'	334 bp	16 rRNA	13
	RNAH2	5'GGTACCGTCAGTCT 3'			
Ureaplasma spp. (biotyping)	MBA-125	5'GTATTTGCAATCTTTATATGTTTTCG3'	408-Biovar1 448- Biovar2	Multiple Banded Antigen	14
	MBA-226	5'CAGCTGATGTAAGTGCAGCATTAAATTC3'			
C. trachomatis	KL1	5'TCCGGAGCGAGTTACGAAGA3'	241 bp	Cryptic Plasmid	15
	KL2	5'AATCAATGCCCGGGATTGGT3'			
C. trachomatis	NLO	5'ATG AAA AAA CTC TTG AAA TCG3'	1128 bp	*Omp1*	16
	NRO	5'CTC AAC TGT AAC TGC GTA TTT3'			

*Multiple x PCR for *Ureaplasma* spp. and *M. hominis*

was not significant [305.00l ± 144.215/μl (155 - 532/μl) vs 345.333 ± 172.339/μl (166 - 621/μl).

Ureaplasma spp. were detected in 6 out of 100 HIV-1 infected patients and one out of 50 of healthy individuals by culture and/or PCR (*P*=0.42). Culture alone detected ureaplasma in four HIV-1 infected patient and one HIV-negative control. *M. hominis* was detected only in one out of 100 (1%) urine samples of the HIV-1 infected patients and in none of the samples from the control group. The positive cultures of *Ureaplasma* spp. and *M. hominis* grew >10^4 CCU/ml after 48 h of incubation. All ureaplasma isolates belonged to biovar1 *(U. parvum)*. All strains of *Ureaplasma* spp. were uniformly susceptible to the four antibiotics tested. MICs for the isolates against each antibiotic were less than 0.5 μg/ml. None of the individuals in the HIV and control groups was tested positive for *M. genitalium*.

C. trachomatis was detected in seven out of 100 (7%) FVU samples of HIV-1 infected adults by PCR and in none of the samples from the healthy control group. All seven samples positive for *C. trachomatis* by cryptic plasmid PCR were confirmed by the PCR targeting the *omp1* gene. Although not statistically significant, frequency of infection with *C. trachomatis* was higher in the HIV-infected population than in the control group. None of the individuals were co-infected with mycoplasmas and *C. trachomatis*.

A statistically significant association was found between CD4 cell count and detection of *C. trachomatis* in HIV-infected adults. It was observed that HIV positive

adults with chlamydia infection had a lower mean CD4 count than those without *C. trachomatis*. [187.857 ± 128.693/μl (48 - 389/μl) vs 354.150 ± 167.737/μl (137 - 612/μl), *P=0.01*] *C. trachomatis* was detected in the urine of four of 19 HIV-1 infected patients with CD4 cell count of < 200/μl compared to three of 81 HIV-1 positive cases with CD4 cell count of >200/μl. Thus a CD4 count <200/μl was identified as a risk factor for infection with *C. trachomatis* in HIV positive patients [OR = 6.93, 95% CI: 1.03-50.82].

The rate of *C. trachomatis* infection in HIV positive adults with younger age (<20 yr) of first sexual exposure was 14.07 per cent (5/34) compared to 3.1 per cent (2/66) in HIV infected individuals with higher age of first sexual exposure (>20 yr) [*P* = 0.03, 95% CI].

Discussion

We attempted to detect mycoplasmas *viz. M. genitalium*, *Ureaplasma* spp. and *M. hominis*, suggested to be co-pathogens in HIV-infection, in the urine of HIV-1 infected individuals. *Ureaplasma* spp. and *M. hominis* were detected in 6 and 1 per cent of HIV-1 infected adults, respectively. In contrast, Hashimoto *et al*[18] found high rates of isolation of *U. urealyticum* (70%) in HIV-positive men which was attributed to the presence of homosexual behaviour and urethritis. The presence of *U. urealyticum* in HIV-1-infected women was evaluated by Linhares *et al*[19] who showed an increased frequency compared to HIV-negative patients and a significant correlation with cervical inflammation. Considering the fact that all HIV-1-

infected adults in our study were asymptomatic for genital tract infection and had no history of homosexual behaviour, a low prevalence of genital mycoplasmas was not surprising.

In our study, the frequency of *Ureaplasma* spp. and *M.hominis* infection was comparable to that observed in healthy subjects. Our findings are in concordance with a previous study[8] in which *U. urealyticum* and *M. hominis* were found in the genital tracts of HIV-1-positive patients at a frequency similar to that reported in HIV-negative adults. In a study by Cordova *et al*[20] no statistically significant difference was observed between the rates of detection of *U. urealyticum* from HIV-infected and HIV-negative STD clinic attendees.

The prevalence of *M. genitalium* infection varies according to geographical region. The reported prevalence of *M. genitalium* in asymptomatic men were 0 per cent in Japan[21], 6 per cent in the UK[22] and 9 per cent in Denmark[23]. The strength of association between HIV and *M. genitalium* infections varies due to the prevalence of HIV cofactors with *M. genitalium*, such as urethritis and homosexual behaviour. The HIV-1-infected adults in our study were asymptomatic for genital tract infections and had no history of homosexual behaviour. Thus, absence of infection with *M. genitalium* was not unlikely. Loubinoux *et al*[24] and Summerton *et al*[25] also failed to detect *M. genitalum* in HIV-infected patients. Costa *et al*[24] detected *M. genitalium* in only 0.9 per cent urine samples from HIV-infected subjects in Sao Paulo. Our findings are similar to the results of Manhas *et al*[27] from India who found that the HIV status of individuals did not affect the frequency of detection of *M. genitalium*.

Martinelli *et al*[6] observed that the frequency of genital mycoplasma infection in non-AIDS HIV patients was comparable to that observed in healthy subjects, but a dramatic increase in frequency of isolation of these organisms was reported in patients with full-blown AIDS. We found no association between the isolation of genital mycoplasmas in HIV-1 infected patients and their mean CD4 counts similar to others[8,19].

Studies have suggested that the difference in frequency of mycoplasma isolation in AIDS patients as compared to non-AIDS patients could be ascribed to colonization by antibiotic-resistant strains in the genital tracts of AIDS patients following broad-spectrum antibiotic therapy commonly received by these patients. This hypothesis was supported by a previous study from Italy[28] where *U. urealyticum* resistance to different antibiotics was observed in full blown AIDS patients. Majority of the HIV-infected individuals enrolled in our study were within one year of diagnosis of their HIV positivity and none had a history of prolonged prophylactic broad-spectrum antibiotic exposure. This probably explains the absence of drug-resistant mycoplasma isolates in our study.

STD screening of HIV clinic attendees has found a high prevalence of *C. trachomatis* in the genital tracts of HIV-positive adults[7,29]. In the present study, the frequency of *C. trachomatis* infection was more in HIV-infected adults than in HIV-negative healthy individuals. Also the detection of *C. trachomatis* was significantly associated with low mean CD4 cell count which implies that colonization of the genital tract with *C. trachomatis* may be dependent on the immune alteration associated with waning CD4+ T-cell count in the course of HIV-infection. Since our study was limited to a small population of HIV-1 infected patients attending a Reference Laboratory of a single hospital, the findings may not be generalized to all HIV-infected adults. To establish the role of CD4 lymphocyte count in genital chlamydia infection in HIV infected individuals, more prospective studies with a larger study population are warranted.

Studies have suggested that STD screening programmes in HIV-infected populations need to be guided by the local epidemiology[7,29]. The observed association of *C. trachomatis* among HIV-infected adults in our study highlights the need for further investigation of this potentially important STI. Proper screening guidelines for systematic testing and treatment of STIs in HIV-infected patients might prove prudent to control the retroviral transmission in developing countries like India.

Conflict of interest: None.

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