Association of genital mycoplasmas including \textit{Mycoplasma genitalium} in HIV infected men with nongonococcal urethritis attending STD & HIV clinics


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\textbf{Background & objectives}: Acute nongonococcal urethritis (NGU) is one of the commonest sexually transmitted infections affecting men. The role of genital mycoplasmas including \textit{Mycoplasma genitalium} in HIV infected men with NGU is still not known. The aim of this study was to determine the isolation pattern/detection of genital mycoplasma including \textit{M. genitalium} in HIV infected men with NGU and to compare it with non HIV infected individuals.

\textbf{Methods}: One hundred male patients with NGU (70 HIV positive, 30 HIV negative) were included in the study. Urethral swabs and urine samples obtained from patients were subjected to semi-quantitative culture for \textit{Mycoplasma hominis} and \textit{Ureaplasama urealyticum}, whereas \textit{M. genitalium} was detected by PCR from urine. The primers MgPa1 and MgPa3 were selected to identify 289 bp product specific for \textit{M. genitalium}. \textit{Chalmeydia trachomatis} antigen detection was carried out by ELISA.

\textbf{Results}: \textit{M. genitalium} and \textit{M. hominis} were detected/isolated in 6 per cent of the cases. \textit{M. genitalium} was more common amongst HIV positive cases (7.1%) as compared to HIV negative cases (3.3%) but difference was not statistically significant. Co-infection of \textit{C. trachomatis} and \textit{U. urealyticum} was found in two HIV positive cases whereas, \textit{C. trachomatis} and \textit{M. hominis} were found to be coinfeciting only one HIV positive individual. \textit{M. genitalium} was found to be infecting the patients as the sole pathogen.

\textbf{Interpretation & conclusions}: Patients with NGU had almost equal risk of being infected with \textit{M. genitalium}, \textit{U. urealyticum} or \textit{M. hominis} irrespective of their HIV status. \textit{M. genitalium} constitutes one of the important causes of NGU besides other genital mycoplasmas.

\textbf{Key words} Genital mycoplasmas - HIV - \textit{Mycoplasma genitalium} - nongonococcal urethritis

Urethritis is a most common presentations amongst men attending the STD clinics\(^1\). In India, the incidence of nongonococcal urethritis (NGU) has been increasing over the past decade\(^1\). \textit{Chlamydia trachomatis} is an established cause of NGU in 20-35 per cent of the cases. \textit{Ureaplasma urealyticum} has long been implicated in the aetiology of acute NGU, but the evidence is still conflicting\(^2,3\). \textit{Mycoplasma hominis} may also cause urethritis in men but to a lesser extent \textit{i.e.}, about 3-4 per cent\(^4\).

\textit{Mycoplasma genitalium} is an important pathogen in male urethritis. Several studies have shown a
significant association between *M. genitalium* and urethritis\textsuperscript{12,13} but none from India. Since its first isolation reported in 1981\textsuperscript{14}, this fastidious and slow growing mycoplasma species has proven to be a difficult organism to isolate by culture. Therefore, detection of this organism has to be relied upon non culture methods like polymerase chain reaction (PCR)\textsuperscript{10,11}.

In HIV infected individuals prevalence and role of genital mycoplasmas is not well studied. It is known that certain mycoplasmas may act as cofactors for the acquisition and progression of HIV disease\textsuperscript{12,13}. They are capable of maintaining low grade infection in male urethra and thus might lead to periodic flare ups in more advanced stages of disease in HIV positive patients. Also, there is evidence that *Mycoplasma* spp. enhance HIV replication rate in peripheral blood mononuclear cells\textsuperscript{12}. Moreover, *Mycoplasma* spp. have been detected with increased frequency in urogenital tracts of HIV positive men without urethral syndromes\textsuperscript{13}.

Therefore, we investigated frequency of isolation/detection of genital mycoplasmas (*M. genitalium*, *U. urealyticum*, *M. hominis*) in HIV infected men with NGU and compared it with HIV uninfected individuals.

**Material & Methods**

**Patients:** Between June 2005 and December 2006, 100 consecutive male patients, suspected to have nongonococcal urethritis (70 HIV positive, 30 HIV negative) attending the HIV and STD clinics of Postgraduate Institute of Medical Education and Research (PGIMER) and Multispeciality Hospital (sector 16), Chandigarh, were enrolled for the study after obtaining informed written consent. The study protocol was approved by the Institute ethics committee.

Cases were defined as men having urethral discharge on examination and urethral inflammation documented by the presence of ≥5 polymorphonuclear cells/high power field with absence of any gonococcus on smear examination after Gram staining and absence of any growth of gonococcus on culture. The HIV serostatus of patients was confirmed as per the guidelines laid down by National AIDS Control Organisation (NACO)\textsuperscript{15}.

**Collection, transport and processing of samples:** Three endourethral swabs and a first void urine sample were taken from all the patients. The swabs were taken with a thin Dacron swab, after cleaning the external urethral meatus with sterile normal saline. First swab was used for smear preparation and microscopic examination, second swab was used for inoculation on to Chocolate agar, for culture of *Neisseria gonorrhoeae*. Males found positive for gonococcus on smear or in culture were excluded from the study (only 2 patients were excluded). Third swab was inoculated into Pleuropneumonia Like Organism (PPLO) broth (Difco, USA) for culture of *M. hominis* and *U. urealyticum*\textsuperscript{15}. Approximately 10-15 ml of urine was collected. After arrival in the laboratory, urine samples were mixed thoroughly and distributed into different aliquots and stored at appropriate temperatures required -70°C for PCR and 4°C for *C. trachomatis*. For mycoplasma culture, 200 µl of urine was used, for DNA extraction 1.8 ml and for detection of *C. trachomatis* 4 ml of urine was used.

**Culture for Mycoplasma hominis and Ureaplasma urealyticum:** From plain PPLO broth used for transport of urethral swabs, 1 ml of the broth was passed into PPLO broth made selective for *M. hominis* by arginine and another 1 ml was passed into PPLO broth made selective for *U. urealyticum* by adding urea as selective agent after filtering it through 0.45 µm syringe filter (Millipore, USA). The tubes were incubated in 5 per cent CO\textsubscript{2}, and were observed thrice daily for any colour change till 15 days. As soon as the colour change to red-pink was seen in the absence of any turbidity of the media, the growth was subcultured to corresponding PPLO agar plates. PPLO agar plates were incubated at 37°C with 5 per cent CO\textsubscript{2} for 15 days. The plates were observed daily under the microscope for any growth till 15 days. Once the colonies appeared, the organism was identified according to standard biochemical methods and staining by Dienes and Giemsa stains as per the standard methods\textsuperscript{15}. For semi-quantitative culture of urine, 200 µl of urine sample was added to 1.8 ml of PPLO broth after filtering it through a 0.45 µm syringe filter (Millipore) and serially 200 µl of this was transferred to subsequent tubes containing 1.8 ml of PPLO broth meant specifically for *M. hominis* and *U. urealyticum*, giving serial ten-fold dilutions starting from 10 to 10\textsuperscript{-5}. The same was incubated at 37°C with 5 per cent CO\textsubscript{2} for 15 days and observed for colour change to red-pink. Once there was a colour change, a subculture was done on to PPLO agar. Typical “fried-egg” colonies were seen. *U. urealyticum* colonies are typically small, up to 50 µm in size whereas the colonies of *M. hominis* are larger, up to 300 µm in size.

The highest dilution which changed the colour of indicator present in the broth represented the number of microorganisms in the sample in colour changing units per ml (CCU/ml). The Mycoplasmas (*Mycoplasma*
hominis and Ureaplasma urealyticum) were identified according to standard methods.15

Polymerase chain reaction (PCR) for M. genitalium: PCR was performed as per the method by Jensen et al.11,16 Briefly, 1.8 ml of urine was centrifuged at 20,000 g for 15 min, supernatant discarded and 300 µl of Chelex 100 slurry (Sigma, USA) [20% w/v Chelex 100 in Tris-EDTA buffer (10 mmol/l Tris-HCl, pH 8.0)] was added. The mixture was vortexed vigorously for 60 seconds and then incubated at 95°C for 10 min. After a brief centrifugation 50 µl of the supernatant was used for PCR.16 Primers MgPa1 (5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3') and MgPa3 (5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3') (Sigma Aldrich Chemicals, USA) were used.

A final reaction volume of 100 µl containing 1X PCR buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl] with 4.5 mM MgCl2; a 0.2 µM concentration of each primer; 1U of Taq polymerase; a 125 µM concentration of dNTP mix and 50 µl of DNA extract was made (Taq polymerase, dNTP mix; Roche Diagnostics, Germany). PCR programme in thermal cycler (Eppendorf, Germany) consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 67°C for 1 min and extension at 72°C for 6 min. All the batches of PCR were run alongwith a positive control using M. genitalium DNA obtained from Statens Serum Institute, Denmark. Amplicons were visualized after electrophoresis on 2 per cent agarose gel stained by ethidium bromide and examined under UV transillumination (Banglore Genei, India) for a 281 bp product11 (Fig.).

ELISA for C. trachomatis infection: For antigen detection of C. trachomatis, ELISA was performed as per manufacturers instructions (Microtrack, Trinity Biotech, Ireland). Briefly, about 4 ml of of urine was centrifuged 2000×g for 15 min and pellet was treated with universal solution as per the package insert supplied in the kit and after boiling step the supernate was used for doing ELISA. The validity of the assay was assessed as per the instructions provided and the cut-off was calculated. All the positive tests were repeated. The sensitivity and specificity of ELISA male urine were 83 and 96 per cent, respectively.

Statistical analysis: The results were compared between the two groups (HIV vs non HIV) and evaluated statistically by Pearson’s chi square test. P<0.05 was considered significant.

Results

Of the 100 symptomatic men, mean age of HIV positive patients with NGU (n=70) was 37.4 ± 6.2 yr and that of HIV negative patients with NGU (n=30) was 31.2 ± 6.4 yr. Dysuria was more common (61%) than urethral discharge (39%).

C. trachomatis antigen was detected in the urine of 21 per cent (21 of 100) of the patients with NGU of which, it was found in 22.8 per cent (16/70) of HIV positive and in 16.6 per cent (5/30) of HIV negative cases with nongonococcal urethritis.

U. urealyticum positive swabs were seen in 18.5 per cent (13 of 70) of HIV positive cases and in 23.3 per cent (7 of 30) of HIV negative cases with NGU. In patients in whom swab culture was positive for U. urealyticum, the urine culture was also positive except for one. However, the quantitative culture of urine was significant (>10⁴ CCU, colour changing units) in 13 per cent cases overall, 11.4 per cent (8 of 70) in HIV positive and in 16.7 per cent (5 of 30) of HIV negative cases.

M. genitalium was detected in 6 per cent (6/100) of NGU cases. The infection rate was 7.1 per cent (5 of 70) among the HIV positive individuals whereas only one HIV negative NGU case was found to be positive (3.3%).

The PPLO broth culture was positive for M. hominis in 11 per cent (11/100) of cases with NGU, being positive in 14.2 per cent (10 of 70) of HIV positive and in 3.3 per cent (1 of 30) of HIV negative cases. All the patients who grew M. hominis on swab
culture also grew the same on urine culture except for one case. The quantitative culture of urine with a significant cut-off of $\geq 10^4$ CCU was positive in 6 per cent (6 of 100), being positive in 7.1 per cent (5 of 70) of HIV positive cases and in 3.3 per cent (1 of 30) of HIV negative cases.

However, with respect to isolation or detection of these organisms (M. genitalium, M. hominis, U. urealyticum, C. trachomatis), no statistically significant difference between HIV positive and HIV negative individuals was found.

Co-infection of C. trachomatis and U. urealyticum was found in two HIV positive cases whereas, C. trachomatis and M. hominis were found in only one HIV positive individual. U. urealyticum and M. hominis was found to be co-infecting four HIV positive and one HIV negative patient. M. genitalium was found to be infecting the patients as the sole pathogen.

**Discussion**

Our data add support to the evidence that M. genitalium along with other genital Mycoplasma spp. is associated with nongonococcal urethritis in men. Moreover, HIV status of the individual does not affect the frequency of detection of M. genitalium, as there was no statistically significant difference in frequency of infection in HIV positive and HIV negative individuals. In this study, M. genitalium was found in 6 per cent of NGU cases. Various studies have found the detection rates of M. genitalium from patients with urethritis ranging from 10 to 54 per cent which is in much higher than our results. Ballard et al. found that M. genitalium was not significantly associated with NGU in either HIV positive or HIV negative men which is similar to what we have also found in the present study. We have also shown that the association of M. genitalium was independent of the presence of C. trachomatis and U. urealyticum. Taylor-Robinson also reported that M. genitalium was found significantly more often in the urethra of men with non-chlamydial nongonococcal urethritis. Amongst asymptomatic subjects, the rates of detection range from none to 17 per cent in various studies.

Among other aetiological agents C. trachomatis (21%) and U. urealyticum (13%) were the organisms most frequently associated with NGU, irrespective of the HIV status of the individuals. The isolation rate of C. trachomatis from nongonococcal urethritis patients varies from 15-40 per cent. The study in South Africa showed that C. trachomatis was found in 13.5 per cent HIV positive cases with NGU whereas its occurrence in HIV negative NGU cases was 5.8 per cent showing no significant association with NGU in either HIV positive or negative men.

U. urealyticum was cultured from urethral swabs of 20 per cent cases of NGU and urine cultures were positive in 13 per cent. No significant association was found between isolation and the HIV status of the patients although the prevalence rate of U. urealyticum has been found to be higher in AIDS patients than in non-AIDS patients. Hashimoto et al. found that in HIV positive individuals the rates of U. urealyticum colonization was 70 per cent, which is much higher than what has been found in the present study. At the same time, lower rate of U. urealyticum isolation, which was 18.9 per cent in asymptomatic HIV positive cases and 13.6 per cent in HIV negative urethritis cases has been found. We also found the co-infection of either or both the mycoplasma species (i.e., M. hominis and U. urealyticum) in 3.3 and 2.9 per cent of HIV negative and HIV positive cases respectively. There was only 1 HIV positive case which had a co-infection with U. urealyticum and C. trachomatis.

There was no significant association of isolation of M. hominis with respect to HIV infection. However, the isolation rate of M. hominis and U. urealyticum by culture of swab was higher than by urine. However, semiquantitative urine culture is a better method as it gives information about the organism load in the male urethra. A study has found that the role of PCR inhibitors was no different in either urethral swab or in first void urine sample. Moreover it has been found in various studies that the organism can be found as a colonizer in healthy males or in HIV positive individuals without any symptoms of urethritis.

In conclusion, our study indicated that patients with NGU had almost equal risk of being infected with M. genitalium, U. urealyticum or M. hominis irrespective of their HIV status. Another aspect of mycoplasma infection which still remains to be addressed, will be the risk of acquiring HIV infection among individuals whose urethra is colonized by mycoplasmas, thus posing a heightened risk of transmissibility. More prospective studies are needed to validate these observations.
needed to establish the role of genital mycoplasmas as relevant pathogens in progression of HIV disease and in AIDS. Besides this, it is suggested that M. genitalium can also cause non-gonococcal urethritis in men apart from C. trachomatis, U. urealyticum and M. hominis. It should be routinely looked for wherever non-gonococcal urethritis is suspected in clinical practice, irrespective of HIV status of the individuals.

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References


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