Diagnosis of common bacterial causes of urethritis in men by Gram stain, culture and multiplex PCR

Ferdush JAHAN MPhil, SM SHAMSUZZAMAN MPhil, PhD, and Sonia AKTER MPhil

Department of Microbiology, Dhaka Medical College, Dhaka

Abstract

Urethritis is one of the most important causes of morbidity and mortality in developing countries. The aim of this study was to detect common bacterial causes of urethritis in men by Gram stain, culture and PCR. 185 male patients who presented at the Skin and venereal clinic of the Dhaka Medical College, Bangladesh with clinical symptoms suggestive of urethritis were enrolled in this study. Urethral discharges were tested for detection of Neisseria gonorrhoeae by Gram stain, culture and PCR. Multiplex PCR assay was done to detect DNA of Chlamydia trachomatis, Ureaplasma urealyticum and Mycoplasma genitalium. Out of 185 participants, 30.27% and 14.6% were infected by Neisseria gonorrhoeae and Chlamydia trachomatis respectively. None of the individuals was found positive for either Ureaplasma urealyticum or Mycoplasma genitalium. Among the Neisseria gonorrhoeae positive patients 27.57% were positive from Gram stain, 26.49% were culture positive, 30.27% were positive by PCR (p<0.001). 32.65% of the Neisseria gonorrhoeae isolates were penicillinase producers and 83.67% were susceptible to ceftriaxone. Considering culture as the gold standard, the sensitivity and specificity of PCR for the detection of Neisseria gonorrhoeae was 100%, and 94.85% respectively with an accuracy of 96.22%. 3.73% of the 134 smear negative and 5.15% of the 136 culture negative samples were positive by PCR. PCR was the most sensitive and rapid method for the diagnosis of urethritis. Multiplex PCR may be a useful approach to laboratory diagnosis of urethritis in men for its high sensitivity and specificity.

Key words: Urethritis, Neisseria gonorrhoeae, Chlamydia trachomatis, Gram stain, culture, PCR, Bangladesh.

INTRODUCTION

Sexually transmitted diseases (STDs) are caused by a large number of diverse microbial agents that are responsible for considerable morbidity and mortality worldwide. Neisseria gonorrhoeae and Chlamydia trachomatis are the two most common bacterial causes of STDs. Globally, 88 million new cases of gonorrhoea occur each year. In Bangladesh, prevalence of Neisseria gonorrhoeae infection are 35.5% - 42%. The total number of new cases of Chlamydial infection is about 101 million. Prevalence of C. trachomatis in India is 30.8% among symptomatic men and women, 12.3% in South Africa among men with urethritis, 25 - 43.5% among sex workers in Bangladesh. Gonorrhoea commonly presents as acute urethritis in men and C. trachomatis frequently infects the urethra. Among the C. trachomatis infected population, 70-80% women and up to 50% men remain asymptomatic. N. gonorrhoeae usually causes symptomatic urethritis among males, and occasionally results in epididymitis.

Microscopy is a fast, cheap and reliable diagnostic method for Neisseria gonorrhoeae in skilled hands, but is usually only reliable for urethral swabs from symptomatic men. Culture has 85-95% sensitivity and a high degree of specificity, but it is expensive and requires personnel trained in handling the fastidious organism. On the other hand, polymerase chain reaction (PCR) provides rapid method of identification within a day and has high sensitivity and specificity. Direct identification of the causative organisms from the specimen by PCR is now revolutionizing the diagnosis of infectious diseases. Therefore, this study is designed to identify common
causative agents of urethritis which include *N. gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma genitalium* directly from urethral discharge by Gram stain, culture and Multiplex PCR.

**MATERIALS AND METHODS**

A cross sectional study was conducted among 185 patients attending Skin and Venereal disease Out Patient Department of Dhaka Medical College Hospital over a period of July 1, 2011 to June 30, 2012. Sexually active male patients with clinical symptoms suggestive of urethritis having urethral discharge were included in this study. This research protocol was approved by Research Review Committee (RRC) and Ethical Review Committee (ERC) of Dhaka Medical College, Dhaka, Bangladesh. Informed written consent was taken from each patient before sample collection. Data regarding age, education status, occupation, high risk behavior such as, history of exposure and frequency of exposure, number of sex partners and history of using condoms were also recorded. Patients who were on antibiotic therapy or had completed antibiotic therapy within the last three days were excluded from the study.

**Sample collection**

Urethral swabs were collected from 185 patients by cleaning around the external urethral meatus using sterile cotton swabs moistened with sterile normal saline and applying gentle digital massage along the line of urethra from above downwards. Three samples of urethral discharge were obtained from each patient. The first swab was immediately used for making a smear on a glass slide. The second swab was used for culture and the third swab was mixed with 2ml of sterile phosphate buffer saline (PBS) and was kept at -20°C until use for PCR.

**Laboratory processing for culture and sensitivity of Neisseria gonorrhoeae**

Presumptive identification of *Neisseria gonorrhoeae* was based on colony morphology, positive oxidase test, positive superoxol test and presence of intracellular gram negative diplococci. The identification was done by sugar utilization test and PCR.

All specimens were examined for the presence of *Neisseria gonorrhoeae* by Gram stained smear and culture on modified Thayer Martin media. Gram negative intracellular diplococci and plenty of pus cells on Gram stained smear were suggestive of *Neisseria gonorrhoeae*. The inoculated plates were incubated at 37°C in a CO2 atmosphere for 24 to 48 hours.

All gonococcal isolates were tested for the production of β-Lactamase (penicillinase) by paper acidometric method. The antimicrobial susceptibility testing was performed in accordance to the CLSI guideline. The antibiotics tested included Penicillin (10 units), tetracycline (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), cefixime (5µg), and cefuroxime (30 µg).

**DNA extraction**

The sample was thawed, then vortexed to make a homogenous suspension and about one ml was transferred into a microcentrifuge tube. It was then centrifuged at 13000 rpm for 10 minutes and supernatant was discarded. After removing the supernatant by aspiration, the pellet was suspended in 100 µl lytic buffer with non ionic detergent (tween 20 (0.45%) and proteinase K (200 µg/ml), incubated at 60°C for 1 hour, then the tube was kept in a block heater (DAIHA Scientific, Seoul, Korea) at 95°C for 10 minutes to inactivate the proteinase K. After centrifugation at 13500 rpm for 10 minutes supernatant was transferred in another microcentrifuge tube and kept in ice or stored at -20°C for PCR amplification.

**DNA amplification**

Oligonucleotide primers were used for PCR amplification of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma genitalium* DNA. *Neisseria gonorrhoeae* DNA was detected by PCR targeting a sequence of cppB gene using primer HO1 (5’GCTACGCATACCCGCGTTGC3’), HO2(5’CGAAGACCTTCGAGCAGAC3’). *Chlamydia trachomatis* DNA was detected by PCR targeting a sequence of cryptic plasmid gene using primer KL1 (5’TCCGGAGCGGAGTACCAAG3’), KL2 (5’ATCAATGCAGGGATTTGC3’). *Ureaplasma urealyticum* DNA was detected by PCR targeting a sequence of urease gene using primer U4 (5’ACGACGTCCATAAGCAACT3’), U5 (5’CAATCTGCTCGTGAAGTATTAC3’). *Mycoplasma genitalium* DNA was detected by PCR targeting a sequence of Adhesin gene using primer MgPa-1(5’AGTTGTGAAACCTTAACCCCTTG3’), MgPa-3(5’CCGTGGAGGGTTTCTTCCATT TTTTGC3’). Size of amplified product of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma genitalium* were 200, 400, 300 and 250 base pairs respectively.
**Ureaplasma urealyticum**, and **Mycoplasma genitalium** were 390bp, 241bp, and 281bp respectively. For each sample, a total 25 μl of mixture was prepared by mixing of 12.5 μl of mastermix (mixture of dNTP, taq polymerase MgCl₂ and PCR buffer), 2 μl of forward primer (Promega Corporation, USA), 2 μl of reverse primer, 2 μl of DNA template and 6.5 μl of sterile distilled water in a PCR tube for *N. gonorrhoeae*. For Multiplex PCR, 25 μl of reaction volume containing 12.5 μl of mastermix, 1 μl of each forward and reverse primer of *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma genitalium*, 2 μl of each DNA template and nuclease free water was added to make up a volume of 25 μl.

All the reagents were placed in a 0.2 ml PCR tube, then centrifuged briefly to remove any bubble. Thirty six cycles of amplification were performed in a DNA thermal cycler (Eppendorf AG, Mastercycler gradient, Hamburg, Germany). Each cycle consisted of a one minute denaturation step at 94°C, 45 second annealing step at 62°C, and one minute thirty second amplification step at 72°C for *N. gonorrhoeae*. For Multiplex PCR, each cycle consisted of one minute denaturation step at 94°C, 45 second annealing step at 55°C, and one minute thirty second amplification step at 72°C. Each reaction was preceded by 10 minute initial denaturation and followed by 10 minute final extension steps. The amplified DNA were loaded into a 1.5% agarose gel, electrophoresed at 100 volts for 30 minutes, stained with 1% ethidium bromide, destained in distilled water for 20 minutes and visualized under UV light (Fig. 1).

**RESULTS**

The mean age of the patients was 27 ± 7 years (range 15-50 years). Among 83 infected patients 54(65.06%) were between 21-40 years of age and 51.81% in 21-30 years. Most of the infected patients worked as drivers (28.57% for gonococcal and 29.63% for chlamydial infection). 55.36% of the gonococcal and 51.85% of the chlamydial infected patients had education status below secondary school level. Most of the infected patients (60.24%) were come from lower income group. Among 56 gonococcal infected patients 32 (57.14%) had extramarital sex, 3 (5.36%) had no extramarital sex and 21 (37.50%) were unmarried. Among 27 chlamydial infected patients 14 (51.85%) had extramarital sex, 2 (7.41%) had no extramarital sex and 11 (40.74%) were unmarried.

Out of 185 participants, 7 participants always used condom and one (14.28%) was infected by *N. gonorrhoeae*. Of 70 participants who used condom occasionally, 14 (20%) were infected by *N. gonorrhoeae* and 9 (12.86%) were infected by *Chlamydia trachomatis*. Of the 108 participants who never used condoms, 41 (37.96%) were infected by *N. gonorrhoeae* and 18 (16.67%) were infected by *Chlamydia trachomatis*. Of the 5 individuals who had...
more than 3 sex partners per week, 3 (60%) and 2 (40%) had gonococcal and chlamydial infections respectively. Among 68 patients who had 2-3 sex partners per week, 38 (55.88%) and 15 (22.06%) patients had gonococcal and chlamydial infections respectively.

_N. gonorrhoeae_ was detected by Gram stain in 27.57% of cases and confirmed by culture in 26.49% of cases. Multiplex PCR was positive for _N. gonorrhoeae_ in 56 (30.27%) cases and for _C. trachomatis_ in 27 (14.6%) cases (Table 1). PCR detected more _Neisseria gonorrhoeae_ than culture and Gram stain (p< 0.001). Multiplex PCR was done to screen for _Ureaplasma urealyticum_, and _Mycoplasma genitalium_ but no sample was found positive. None of the samples were simultaneously positive for _N. gonorrhoeae_ with _Chlamydia trachomatis_. Among 114 suspected cases who had history of exposure, 68 (59.65%) cases were found infected; of them 47 (41.23%) cases and 21 (18.42%) cases were infected by _N. gonorrhoeae_ and _C. trachomatis_ respectively. Out of 71 cases who had no history of exposure, 15 (21.13%) cases were found infected; of them 9 (12.68%) cases and 6 (8.45%) cases were infected by _N. gonorrhoeae_ and _C. trachomatis_ respectively (Table 2).

Of the 185 samples, 49 were positive by both PCR and culture, 7 of the 136 culture negative cases were positive by PCR (Table 3). Considering culture as gold standard, the sensitivity of PCR was 100%, specificity was 94.85%, positive predictive value was 87.5%, negative predictive value was 100% and accuracy was 96.22%. Among the isolated _N. gonorrhoeae_, 83.67% were susceptible to ceftriaxone; 75.51% to cefixime and 55.10% to ciprofloxacin. The resistance rates to ciprofloxacin and tetracycline were 44.90% and 46.94% respectively.

### DISCUSSION

The aim of this study was to establish a rapid diagnostic test for the detection of different microbial agents causing urethritis among 185 clinically suspected male with urethritis. In this study, the rate of gonococcal infection was 30.27% among 185 sexually active male patients. The rate of infection with _N. gonorrhoeae_ in this study was consistent with previous studies conducted among commercial sex workers which found that the rate of isolation of _N. gonorrhoeae_ was 35.8% and 35.5% among hotel-based and street-based sex workers respectively.7,8 In contrast, a much lower rate of 11% of the 669 patients were positive for gonococcal infection in another study.24 The difference in gonococcal infection rates reflect that the infection varies in different population, regions, social background and sexual behaviours of the patients.

In this study _Chlamydia trachomatis_ was detected in 14.6% of patients with urethral discharge. _Chlamydial_ infection was positive in 12.3% of the patients having urethral discharge in South Africa10 which was in agreement with the finding of the present study. In the present study, neither _Ureaplasma urealyticum_ nor _Mycoplasma genitalium_ was detected by PCR. In one study, the detection rate of _M. genitalium_ was 10% but no _U. urealyticum_ was detected among that African population.25 In another study in Teheran, the reported prevalence of _M. genitalium_ was 7.2% and _U. urealyticum_ was 19.2%.26

### TABLE 1: Different methods for detection of _N. gonorrhoeae_ and _C. trachomatis_ among the suspected cases (n=185)

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive cases n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>51 (27.57)</td>
</tr>
<tr>
<td>Culture</td>
<td>49 (26.49)</td>
</tr>
<tr>
<td>Multiplex PCR N.gonorrhoeae</td>
<td>56 (30.27)</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>27 (14.6)</td>
</tr>
</tbody>
</table>

### TABLE 2: History of sexual exposure and rate of gonococcal and chlamydial infections

<table>
<thead>
<tr>
<th>History of exposure</th>
<th>Suspected cases n(%)</th>
<th>Gonococcal infection n(%)</th>
<th>Chlamydial infection n(%)</th>
<th>Total infected patients n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>114 (61.62)</td>
<td>47 (41.23)</td>
<td>21 (18.42)</td>
<td>68 (59.65)</td>
</tr>
<tr>
<td>Absent</td>
<td>71 (38.38)</td>
<td>9 (12.68)</td>
<td>6 (8.45)</td>
<td>15 (21.13)</td>
</tr>
<tr>
<td>Total</td>
<td>185 (100.00)</td>
<td>56 (53.91)</td>
<td>27 (26.87)</td>
<td>83 (80.78)</td>
</tr>
</tbody>
</table>
MULTIPEX PCR FOR URETHRITIS

In the present study, among the isolated *N. gonorrhoeae*, the sensitivity to ceftriaxone was excellent with 83.67% were sensitive.

Out of 185 cases, 56 *N. gonorrhoeae* cases were diagnosed by PCR, 49 were diagnosed by culture and 7 were positive by PCR but negative by culture. These 7 PCR positive culture negative samples might be due to presence of dead bacteria or presence of inhibitory substances in urethral discharge that hampered with their growth. Considering culture as “gold standard” the sensitivity of PCR was 100% and specificity was 94.85%, positive predictive value was 87.5%, Negative predictive value was 100% and accuracy was 96.22%. The difference in positivity between culture and PCR was statistically significant (p< 0.001). Previous studies had shown the sensitivities ranged between 96.6% to 100%.27,28

In the present study, 14.28% of the gonorrhoea patients gave history that they always used condom during sex. In another study, gonococcal infection among the patients who reported 100% condom use was 8% versus 23% among the patients who reported irregular condom use.29 This finding is in agreement with the finding of the present study. A few among the the cases acquired infection in spite of condom use regularly and this failure might be due to 1) inappropriate use of condoms that may allow the organisms to reach the urethra for infection 2) answering falsely to the interviewer to get positive response of healthcare and 3) rupture of condom. There has been sufficient evidence that condoms when used correctly and consistently, are effective in protection against the transmission of all STIs including gonococcal infection.

The accurate detection of the causative agents of urethritis is necessary because the treatment option for gonococcal urethritis is different from nongonococcal urethritis. The findings of the present study suggest that multiplex PCR assays are potentially useful tools for the rapid detection of *N. gonorrhoeae, C. trachomatis, Ureaplasma urealyticum,* and *Mycoplasma genitalium* from urethral discharge in men with urethritis.

Multiplex PCR is most rapid and sensitive technique for the diagnosis of gonococcal and nongonococcal urethritis. Multiplex PCR may be recommended especially in clinically suspected patients who remain negative by conventional methods. We recommend that further study should also be done in Bangladesh to further identify *Mycoplasma genitalium* and *Ureaplasma urealyticum* that could not be detected in the present study as these organisms are known to cause nongonococcal urethritis.

ACKNOWLEDGEMENTS

The authors thank to Department of Skin and Venereal disease of Dhaka Medical College Hospital for providing data collection. We gratefully acknowledge to Department of Microbiology of Dhaka Medical College for technical help.

REFERENCES


2. Johnson RE, Newhall WJ, Papp JR. Screening tests to detect *Chlamydia trachomatis* and *Neisseria