DETECTION OF COMMON BACTERIAL CAUSES OF URETHRITIS IN SYMPTOMATIC MEN AT THE STD LABORATORY OF MU-SOFIA BY MICROSCOPY, CULTURE AND NAAT

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ABSTRACT

INTRODUCTION: Urethritis is one of the major causes of morbidity in men. The primary pathogens are *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, but also *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Trichomonas vaginalis*, anaerobes, *Herpes simplex virus* (HSV), and adenovirus.

AIM: The aim of this study was to detect common bacterial causes of urethritis in symptomatic men by Gram stain, culture and nucleic acid amplification techniques (NAAT), and to compare them.

MATERIALS AND METHODS: Seventy-eight male patients with clinical symptoms suggestive of urethritis were enrolled in the study. Three urethral samples were obtained from each one - for smear on a glass slide, culture, and NAAT. The glass slides were subjected to Gram stain. Culture on modified Thayer Martin media was used for detection of *N. gonorrhoeae*. Isolation of *C. trachomatis* was performed on McCoy cells, afterwards stained for immunofluorescence with anti-lipopolysaccharide monoclonal antibody. Cultivation and enumeration of *Ureaplasma spp.* and *M. hominis* was done with Mycofast Revolution kit.

DNA extractions and amplifications by using multiplex Real Time PCR tests were done for all the bacteria.

RESULTS: In 30 persons, infections were detected by using different microbiological tests. *N. gonorrhoeae* was discovered by Gram stain in 5 samples; by cultivation - in 6; by PCR - in 8. *C. trachomatis* was found in 13 samples with cultivation; by PCR - in 14. *Ureaplasma spp.* was found in 7 samples with cultivation and in 9 with PCR. *M. genitalium* was detected only by PCR in 3 samples. *M. hominis* was negative in all tested swabs. Co-infections with two microorganisms were detected in 4 samples. All the samples with positive results showed increased number of leukocytes on Gram staining.

DISCUSSION: Although many infections can be diagnosed on the basis of clinical criteria alone, accurate and timely diagnostic microbiology is essential for the clinical management of patients' infections. In our study the PCR was the most sensitive and rapid method for the diagnosis of urethritis in symptomatic men. Nevertheless we recommend that the test is combined with at least one more technique for greater accuracy in the interpretation of the results.

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INTRODUCTION

Urethritis, or inflammation of the urethra, is one of the major causes of morbidity in men. Typical symptoms are urethral discharge, penile stinging/itching, penile tip irritation and dysuria. Up to half of the cases of urethritis are non-specific or asymptomatic, so commonly remain undiagnosed (1,2). In the majority of cases it is caused by sexually transmitted agents. When *Neisseria gonorrhoeae* is detected, the urethritis is referred as gonococcal, and in the other cases - as non-gonococcal (NGU). A number of other pathogens have been implicated in non-gonococcal urethritis - *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Trichomonas vaginalis*, anaerobes, *Herpes simplex virus* (HSV), and adenovirus. Because of the life specificities of these microorganisms, different microbiological diagnostic procedures for accurate diagnosis are needed - microscopy, cultivation, and nucleic acid amplification techniques (NAAT).

AIM

The aim of this study was to detect common bacterial causes of urethritis in symptomatic men and their prevalence by Gram stain, culture and NAAT, and to compare the results.

MATERIALS AND METHODS

A cross-sectional study was conducted among 78 patients attending the Department of Medical Microbiology, MU-Sofia. Sexually active patients with clinical symptoms suggestive of urethritis having urethral discharge were included in the study. Informed written consent was taken from each patient before sample collection. Patients on antibiotic therapy within the last four weeks were excluded from the study.

Sample Collection

Urethral swabs were taken from all the 78 patients by using sterile cotton tip moistened with sterile normal saline. Three samples of the urethra were obtained from each patient. The first swab was used for preparing a smear on a glass slide stained afterwards with Gram staining. The second swab was used for culture, and the third swab was suspended in sterile phosphate buffer saline (PBS) and was kept at -20°C until DNA extraction.

Laboratory Processing for *Neisseria gonorrhoeae* Detection

All specimens were examined for the presence of *N. gonorrhoeae* by Gram-stained smear and culture on modified Thayer Martin media. Gram-negative intracellular diplococci and plenty of leukocytes (more than 30) cells on Gram-stained smear were suggestive of acute *N. gonorrhoeae*. Extracellular diplococci with concentration of leukocytes between 10-30 were suggestive of chronic infection. The inoculated plates were incubated at 37°C in a CO₂ atmosphere for 24 to 48 hours. The identification of *N. gonorrhoeae* was based on colony morphology and positive oxidase test. The final identification was performed by PCR.

Laboratory Processing for *Chlamydia trachomatis* Detection

Isolation of *C. trachomatis* in cell culture was performed. Bacteria in the samples were detected by culturing method on McCoy cells and stained for immunofluorescence with anti-lipopolysaccharide monoclonal antibody according to all the rules of indirect immunofluorescence. The monoclonal antibody used - Mab 202D7, was aimed at the group-specific lipopolysaccharide antigen of chlamydiae. This antibody has been prepared, studied and characterized in our lab (3). Subsequently, coverslips monolayers were stained with Evans-blue and examined for chlamydial elementary bodies by means of a fluorescence microscope. Reference strains and monoclonal antibodies were used for quality control. Both positive and negative controls were used in parallel for the cell-culture method.

Polymerase chain reaction was performed as confirmatory and very specific test.

Laboratory Processing for *M. hominis*, *M. genitalium* and *Ureaplasma spp*

All the samples were tested for the presence of *M. hominis*, *M. genitalium* and *Ureaplasma spp*. Two different methods were used. The first was cultivation and enumeration of *U. urealyticum* and *M. hominis* with Mycofast Revolution kit (ELITech, France). The samples were analyzed according to the manufacturer's requirements. The second test for detection of genital mycoplasmas and ureaplasmas was PCR based technique.

DNA Extraction and Amplification by Using Multiplex Real Time PCR Tests

The final identification of the specimens was performed by PCR.

Each urethral sample was diluted in the 2SP medium, vortexed and after this subjected to a DNA extraction process using DNA-Sorb-A test (Sacace Biotechnologies Srl, via Scalabrini 44-22100-Como-Italy) according to the manufacturer's instructions. The extracted DNA was used directly for PCR amplification or kept in ice and stored at -20°C.

Detection of *C. trachomatis*, *U. urealyticum* and *M. hominis*; as well as *N. gonorrhoeae*, *C. trachomatis*, and *M. genitalium* was performed with mRT-PCR kit (Sacace Biotechnologies Srl, via Scalabrini 44-22100-Como-Italy) using LineGene.K (Bioer Technology Co., Ltd., Bio Flux Corporation, Tokyo, Japan) machine. The procedure followed the manufacturer's requirements.

RESULTS

A total of 78 sexually active men with clinical symptoms suggestive of urethritis having urethral discharge were tested in the STD laboratory of MU-Sofia. Three urethral samples were obtained from each one – the first for smear on a glass slide and subsequent Gram staining, the second – for culture on selective media, and the last - for DNA extraction and PCR. In 30 persons mono- or co-infections were detected by using different microbiological tests. *N. gonorrhoeae* was discovered by using Gram stain in 5 samples; by cultivation on modified Thayer Martin media in 6 swabs; and by PCR in 8 smears. *C. trachomatis* positive results were obtained by performing cultivation on McCoy cells and subsequent immunofluorescence with anti-lipopolysaccharide monoclonal antibody in 13 samples; by using PCR the positive results were 14. *U. urealyticum* was found in 7 samples with the cultivation and enumeration with Mycofast Revolution kit; and in 9 swabs by performing PCR technique. *M. genitalium* was detected in 3 cases only by applying PCR. *M. hominis* was negative in all tested swabs. Co-infections with two microorganisms were detected in 4 samples and the combinations were as follows: *C. trachomatis* and *N. gonorrhoeae* in 1 sample; *C. trachomatis* and *M. genitalium* in 1 sample; *C. trachomatis* and *Ureaplasma spp.* in 2 samples. All the sam-

ples with positive results showed increased number of leukocytes on Gram staining.

DISCUSSION

The aim of this study was to establish a diagnostic test for the detection of different microbial agents causing urethritis among 87 clinically suspected male with urethritis.

Acute NGU is one of the commonest STIs affecting men. Symptoms of urethritis in men typically include dysuria, urethral discharge, penile itching or tingling. A diagnosis can be suspected if at least one of the following is present: discharge, a positive result on a leukocyte esterase test in first void urine, or at least 10 white blood cells per high-power field in urine sediment (4). History and examination findings can help distinguish urethritis from other urogenital syndromes, such as epididymitis, orchitis, and prostatitis. But exact diagnosis and precise treatment could be achieved only if pathogen or combination of pathogens are detected.

The primary microorganisms associated with urethritis are *C. trachomatis* and *N. gonorrhoeae* (4). The classification of urethritis as gonococcal or non-gonococcal is based on the traditional Gram staining of urethral discharge for gram-negative diplococci. But gonococcal and non-gonococcal infections often co-exist. This means the classification is not accurate.

Other common reasons for urethritis are *M. genitalium* and *U. urealyticum*. Not so common but important enough are *T. vaginalis*, anaerobes, *Herpes simplex virus* (HSV) and adenovirus (5). That was the reason to perform study in which to detect the primary pathogens of urethritis - *N. gonorrhoeae*, *C. trachomatis*, *M. genitalium* and *Ureaplasma spp.*

The etiological diagnosis of infectious diseases, including urethritis, relies on the microbiological examinations. And there are different methods that could be applied.

The microscopic examination of clinical specimens has been well known as the first to be performed in diagnostic microbiology. The microscopy technique can be done routinely in a variety of clinical settings. Direct microscopy may show the specimen's quality, detecting a variety of organisms in the clinical specimen, finding out epithelial cells, and evalu-

ating the type of inflammatory response according to the presence of leukocytes. More information is acquired, if some staining is used. Gram staining is the procedure more commonly applied in routine labs. Typical Gram reactions, morphologies and arrangements of the observed organisms and cells give the presumptive identification of some certain etiological pathogens. Furthermore, the constellation analyzed by the microscopy may also guide the specialist in selecting appropriate isolation media and culture methods afterwards, and aid the physician in selecting the empirical antibiotic therapy. That is the reason we started our investigation with this procedure.

According to it 34 samples showed increased number of leukocytes.

Microscopy could be used as a fast, cheap and reliable diagnostic method for *N. gonorrhoeae* in skilled hands. By using it, 5 samples from the 78 tested swabs resulted positive. However, it is good to be known that usually only urethral swabs from symptomatic men could be so informative by using the microscopy technique (6). One more disadvantage of the method is its inability to identify the species. That is why we continued our work with cultivation of the samples on selective media.

Although gonorrhea could be easily diagnosed by inspection of yellowish discharge going out from the urethra, many patients infected with *N. gonorrhoeae* show no discharge. Furthermore, around 10% of infected males are asymptomatic. So, culture of the samples on modified Thayer Martin media was performed for detection of *N. gonorrhoeae* (7). The inoculated plates with the patient's samples were incubated at 37°C in a CO₂ atmosphere for 24 to 48 hours. The identification of *N. gonorrhoeae* colonies was based on their morphology, positive oxidase test, and sugar utilization using Quadferm (bioMérieux Vitek, Inc., Hazelwood, Mo.). With this method the 78 tested samples showed positive result in 6 patients. The cultivation method gave us the possibility to test the strain's sensitivity toward different antibiotics. Culture showed better sensitivity and was with higher degree of specificity in comparison with the Gram staining, but it is more expensive and requires personnel trained in handling the fastidious organism (8). Another disadvantage of the method is the need of more days for the cultivation process. Some-

thing else, gonorrhea-selective culture medium has progressively developed, but the antibiotics which have contributed to the improvement may occasionally inhibit certain sensitive strains (9).

With the PCR method the positive results for *N. gonorrhoeae* in our study were 8. The molecular diagnosis of the infection is much more rapid, but it is expensive and requires well trained personnel (10). But it does not always give better results. Serra-Pladevall J et al. have done a comparison between conventional culture and NAATs for the microbiological diagnosis in gonococcal infection. They have showed that sensitivity, specificity, positive predictive value, and negative predictive value for culture are 86.2%, 99.8%, 99.2%, and 96.7%, respectively, and for PCR - 98.7%, 100%, 100% and 99.7%, respectively (11). So, we think that in laboratories, especially where antimicrobial susceptibility is monitored, an effective approach would be to perform culture in addition to PCR in symptomatic patients suggestive for gonococcal infection.

The most common reason for the so-called NGU is *C. trachomatis*. The microorganism is observed in 30 to 40% of men with this diagnosis (12). However, about half of the infected subjects are asymptomatic. In addition, *C. trachomatis* could not be detected by routine microbiological testing (13, 14). This is because they are intracellular and very fastidious. For the detection of *C. trachomatis* two procedures are recommended – cultivation on selective media with cell lines (the gold standard) and/or NAATs (15). In our study we performed both. First we did cultivation on McCoy cell line followed by immunofluorescence with anti-lipopolysaccharide monoclonal antibody. By using this methodology we found 13 *C. trachomatis* positive samples. Afterwards we did PCR with which method the positive samples resulted 14. The reason for the reduced number of proved by cultivation positive results in comparison with the PCR technique could be the fact that sometimes the patient's specimen is toxic for the used McCoy cell culture line and the inability of the bacterium to grow up in it (16).

It is still unknown whether *Mycoplasma* and *Ureaplasma* species are common in men with urethral symptoms (17). *U. urealyticum* has been recognized as a pathogen for NGU since the 1950s (18).

Nevertheless, the exact role of the both groups of microorganisms as pathogens is still controversial (19). For this reason we decided to test the samples for their presence. For *M. hominis* and *Ureaplasma spp.* cultivation and enumeration Mycofast Revolution kit (ELITech, France) was used. The samples were analyzed according to the manufacturer's requirements. With cultivation on selective media not a single specimen was assigned as positive for *M. hominis* but there were 7 for *Ureaplasma spp.* According Carlin et al., the culture method has 85-95% sensitivity and a high degree of specificity, and requires personnel trained in handling the fastidious organism (20). When using the PCR method, the *Ureaplasma spp.* positive samples were 9 and again *M. hominis* was not detected.

The role of *M. genitalium* in NGU urethritis has attracted much attention in the past 10-15 years. However, the microorganism still remains uncultivated. Nevertheless, many studies have concluded that *M. genitalium* is a common cause of non-gonococcal urethritis and that eradication is associated with symptomatic improvement (21, 22). These studies rely on the detection of the bacterium by using molecular techniques. In the present study, from the 87 tested samples, 3 showed positive results after the application of a PCR test.

It is clearly seen that direct identification of the causative organisms from the specimen by PCR is now revolutionizing the diagnosis of infectious diseases. NAATs are more and more frequently used in many medical fields. The reason is that PCR tests provide a rapid method of identification within a day and have high sensitivity and specificity. They are preferred assays because of the ability to screen with noninvasive specimens such as urine (23). Multiplex PCR is one more step in the rapid and sensitive technique for the diagnosis of different infectious diseases, including gonococcal and nongonococcal urethritis. Multiplex PCR may be recommended, especially in clinically suspected patients who remain negative by conventional methods.

The accurate detection of the causative agents of urethritis is necessary, because the treatment option for gonococcal urethritis is different from non-gonococcal 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 spp.*, and *M. genitalium* from urethral discharge in men with urethritis.

CONCLUSION

Although many infections can be diagnosed on the basis of clinical criteria alone, accurate and timely diagnostic microbiology is essential for the clinical management of patients' infections. In our study PCR was the most sensitive and rapid method for the diagnosis of urethritis in symptomatic men. Nevertheless, we recommend that the test is combined with at least one more technique for greater accuracy in the interpretation of the results.

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