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Distribution of sexually transmitted diseases in a group of symptomatic male patients using urine samples and PCR technique

Distribuția bolilor cu transmitere sexuală într-un grup de barbați simptomatici utilizand probe de urina si tehnica PCR

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Abstract

Sexually transmitted diseases (STDs) are a very important cause of illness worldwide and prolonged, untreated infections with STD pathogens may have serious consequences. Our study aims to evaluate the distribution of six different STDs (Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Ureaplasma urealyticum, Mycoplasma hominis and Mycoplasma genitalium) in male urine samples. First void urine samples from 52 symptomatic patients were collected between April 2014 and April 2015. DNA was extracted, purified and amplified via multiplex polymerase chain reaction (PCR) for the detection of the six STD pathogens, further identified using a 2% agarose gel electrophoresis with ethidium bromide as staining agent. STD frequency in the study group was 53.84 % (28 patients), mostly in the 20-29 years age group. Among positive patients, six presented multiple infections. 35 positive DNA samples were identified in the study: 17 of C. trachomatis, 9 of U. urealyticum, 7 of N. gonorrhoeae and 2 of M. genitalium. Wide scale application of the system based on the simultaneous detection of these six pathogens inducing STD may facilitate diagnosis, especially in multiple infections.

Keywords*: STD pathogens;DNA;simultaneous detection;PCR;gel electophoresis*

Rezumat

Bolile cu transmitere sexuală (BTS) reprezintă o cauză foarte importantă a îmbolnăvirilor în lume, iar infecţiile prelungite şi netratate cu agenţii patogeni care provoacă BTS pot avea consecinţe serioase. Scopul studiului nostru a fost să evalueze distribuţia a şase tipuri diferite de BTS (Chlamydia trachomatis, Neisseria gonorrhoeae,

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Trichomonas vaginalis, Ureaplasma urealyticum, Mycoplasma hominis şi Mycoplasma genitalium) în probe de urină recoltate de la bărbaţi. Intre aprilie 2014 şi aprilie 2015 s-a colectat primul jet de urină de la 52 de pacienţi simptomatici. S-a extras ADN-ul, s-a purificat şi s-a amplificat prin tehnica polymerase chain reaction (PCR) pentru detecţia simultană a agenţilor patogeni menţionaţi, iar pentru identificare s-a utilizat electoforeza în gel de agaroză 2% cu bromură de etidiu ca agent de colorare. Frecvenţa BTS în grupul luat în studiu a fost de 53.84% (28 de pacienţi), cei mai mulţi în grupa de vârstă 20-29 ani. Dintre pacienţii pozitivi, şase au prezentat infecţii multiple. S-au detectat 35 probe de ADN pozitive: 17 de C. trachomatis, 9 de U. urealyticum, 7 de N. gonorrhoeae şi 2 de M. genitalium. Aplicarea pe scară largă a sistemului bazat pe detecţia simultană a acestor şase agenţi patogeni care induc BTS poate să faciliteze diagnosticul, în special în infecţiile multiple.

***Cuvinte cheie**: patogeni BTS;ADN;detectie simultana;PCR;electoforeza*

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Introduction

There are over 25 infectious organisms that cause sexually transmitted diseases (STDs) - bacterial, viral and parasitic infections acquired through sexual activity.

STDs are a topical issue in developing countries, posing a serious socio-economic impact. In many developing countries there are no appropriate laboratory methods for the diagnosis of sexually transmitted diseases. World Health Organization (WHO) recommends treatment of STDs following some proposed algorithms, depending on STD syndromes (1). An appropriate treatment requires accurate detection of the pathogens causing STDs.

According to the Centers for Disease Control and Prevention (CDC) statistics, an estimated 19 million new STD cases occur each year, almost half of them in the 15-to-24-year age group (2). WHO 2008 statistics revealed that about 498.9 million new adult cases of *Chlamydia trachomatis, Neisseria gonorrhoeae*, Syphilis and *Trichomonas vaginalis* occurred worldwide (3).

Certain behavioral factors such as unprotected sex and multiple partners are known to increase the risk of STD acquisition. Biological factors that may influence STD acquisition include age, co-infection with other sexually transmitted diseases and hormonal contraceptive use (4, 5, 6).

Infertility is a significant problem worldwide and, according to some authors, STDs are a cause of unexplained infertility. Screening and treatment of STDs in patients with unexplained infertility may increase the pregnancy rate (7).

A number of biologic, cultural and socioeconomic factors contribute to a much higher incidence and prevalence of S'I'D infection in women as compared to men. Because the vagina presents a large mucosal area and a suitable environment for bacterial growth and multiplication, women are more likely to get infected. Some authors indicate that in several infections, e.g. gonorrhea and chlamydia, the incidence of infected males transmitting STD pathogens to healthy females during intercourse is higher than the other way around (8, 9). Prolonged, untreated infections with these pathogens may have serious consequences for male subjects, resulting in urethritis, prostatitis or epididymitis. It is known that microbiota exists in the lower male genital tract but there are insufficient data associating male microbiota with STD acquisition (10).

Because there is a large number of STD pathogens which can generate co-infections, their simultaneous detection in the same clinical sample is very important. Polymerase chain reaction (PCR) is an advanced molecular biology method, presenting high sensitivity and rapidity compared to traditional methods (culture-based and other standard methods) (11, 12). Quick detection of these pathogens is important and mul-

tiplex PCR is a rapid assay that can be completed in a few hours. Isolation in cultures may take up to 4 days in case of *Ureaplasma urealyticum* and *Mycoplasma hominis* and as long as 7 days for *T. vaginalis* (13). On the other hand, in bacterial cultures only viable bacteria can be quantified, while nucleic acid amplification tests such as PCR can quantify both viable and nonviable bacteria (14). Simultaneous detection of multiple pathogens using PCR amplification presents higher sensitivity and specificity compared to reference methods (14, 15).

Our study aimed to evaluate the distribution of six different types of STDs (*C. trachomatis, N. gonorrhoeae, T. vaginalis, U. urealyticum, M. hominis* and *M. genitalium*) in urine samples collected from symptomatic male patients.

Material and methods

Patients and specimens

A number of 52 male patients aged 22 to 71 years were enrolled in this study between April 2014 and April 2015. All persons presented acute or chronic STD symptoms such as genital discharge, dysuria, fever and abdominal pain and were recruited multi-disciplinarily from the dermatology, urology and infectious diseases' ambulatory departments in Cluj-Napoca. The research protocol (no. 146/15.04.2014) was approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca. None of the patients were given antibiotic treatment two weeks prior to their inclusion in the study. Age distribution of the 52 patients is shown in Figure 1.

For all patients 30 to 50 mL of first void urine (FVU) samples were collected in sterile polypropylene containers during early morning hours, provided they had not urinated for at least 4 hours.

Pretreatment of Clinical Specimens and DNA Extraction

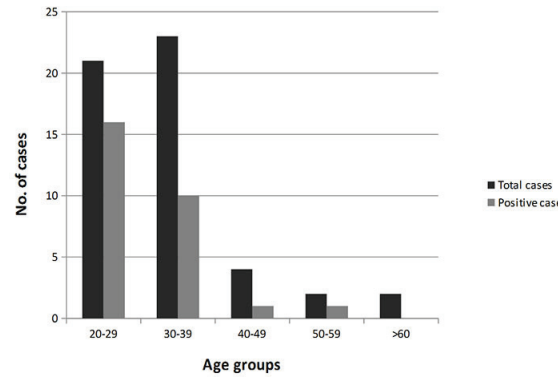

Figure 1. Age distribution of patients / positive patients

Urine was refrigerated at 2-8°C and brought up to room temperature before DNA extraction. To avoid freezing, sample collection and DNA extraction were carried out in the same week. Aliquots of urine samples were centrifuged for 15 minutes at 15,000 g and most of the supernatant was removed to increase urine concentration.

For DNA extraction and purification we used MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE Biotechnologies, USA). 150 µL of concentrated urine samples were transferred into micro-centrifuge tubes with tissue and cell lysis solution containing proteinase K, and were thoroughly mixed. The mix was incubated for 15 minutes at 65°C and then cooled to 37°C, when RN-ase A was added and mixed thoroughly. The sample was further incubated for 30 minutes at 37°C and then placed on ice for 5 minutes. MPC Protein Precipitation Reagent was added to the lysed sample and the mix was vortexed vigorously. The debris were pelleted through micro-centrifugation at 14,000 g for 10 minutes and the supernatant was transferred into a clean tube where isopropyl alcohol was added for DNA precipitation. The DNA pellet was separated by centrifugation and the isopropyl alcohol was poured off. The DNA pellet was then rinsed twice with 75% ethanol and re-suspended in TE buffer.

DNA concentration and purity were determined via nano-photometric readings against a reference TE buffer solution. When DNA concentration was not high enough, the DNA extraction was repeated using larger sample aliquots. The A260/280 ratio (corresponding to absorbance values at 260 and 280 nm) and A260/230 ratio (corresponding to absorbance values at 260 and 230 nm) were determined via spectrophotometric measurements, thus assessing DNA purity. When purity was not appropriate, the DNA purification sequence (dilution in tissue and cell lysis solution, protein precipitation with MPC Protein Precipitation Reagent, DNA precipitation with isopropyl alcohol and rinses with 75% ethanol, DNA pellet separation via centrifugation and re-suspension in TE buffer) was repeated.

Polymerase chain reaction (PCR)

The amplification reaction was carried out with Seeplex® STD6 ACE Detection (Seegene, Korea, certified IVD) in a total volume of 20 µl including 3 µl DNA extract and 17 µl PCR mix with 5x STD6ACE PM primers containing primer pairs for *C. trachomatis, N. gonorrhoeae, T. vaginalis, U. urealyticum, M. hominis* and *M. genitalium*, a primer pair for internal control and an internal control template.

The mixture was submitted to 40 amplification cycles on a DNA thermal-cycler. PCR was initiated with a 15 minutes denaturation step at 94°C and finished with a 10 minutes extension step at 72°C. Each cycle included a denaturation step at 94°C (30 seconds), an annealing step at 63°C (90 seconds) and a chain elongation step at 72°C (90 seconds).

Agarose Gel Electrophoresis

A 2% agarose gel containing ethidium bromide was used with 0.5X Tris-borate 0,1 mM EDTA (TBE) as running buffer. The wells were loaded as follows: 5 µL negative control, 5 µL of each sample, 5 µL positive control, 5 µL STD6 ACE marker used to determine the approximate size of the targeted products. With the electrophoresis source set at 100 V, voltage was applied for 45-60 minutes. The PCR products were visualized using a UV transilluminator.

Statistical analysis

The *chi square* (χ^2) test can be used to evaluate a relationship between two categorical variables. In our study it was used to determine whether there was a significant difference between the theoretically expected occurrence frequencies of STD pathogens and the observed frequencies (actual results).

Results

Migration of PCR products on one of the agar gels is shown in Figure 2. Table I presents the number and frequency of the positive results detected in the 52 cases of male STD-symptomatic patients.

As shown, 28 (53.84%) of the 52 patients enrolled in our study presented STD infections. 35 positive DNA samples were identified overall: 17 of *C. trachomatis*, 9 of *U. urealyticum*, 7 of *N. gonorrhoeae* and 2 of *M. genitalium*. Distri-

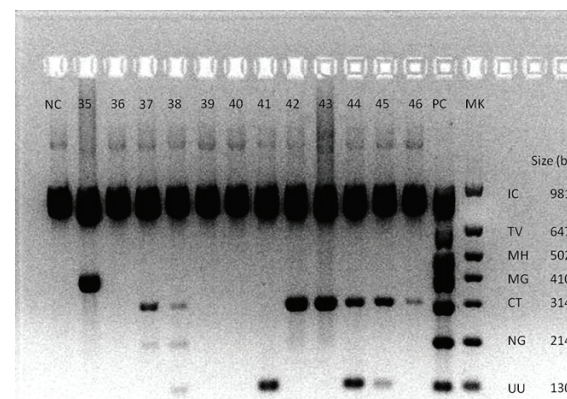

Figure 2. STD pathogens - agarose gel electrophoresis. NC: negative control, 35-46: samples, PC: positive control, MK: marker, TV: *T. vaginalis*, **MH:** *M. hominis*, **MG:** *M. genitalium*, **CT:** *C. trachomatis,* **NG:** *N. gonorrhoeae,* **UU:** *U. Urealyticum*, **bp: base pairs**

Table I. Presence of STD pathogens in the positive samples

Organism	No. of positive samples (N=52)	Frequency (%)
C. trachomatis	17	32.69
U. urealyticum	9	17.31
N. gonorrhoeae	7	13.46
M. genitalium	2	3.85
Single microorganism detected	22	42.31
Multiple microorganisms detected	6	11.54
All negative results	24	46.15

bution of STD agents in the positive samples can be observed in Figure 3.

Five patients have been found with double infection: *C. trachomatis* and *N. gonorrhoeae* – 2 patients, *C. trachomatis* and *U. urealyticum* – 2 patients, *N. gonorrhoeae* and *U. urealyticum* – 1 patient. One patient has been found with triple infection: *C. trachomatis, N. gonorrhoeae* and *U. urealyticum.*

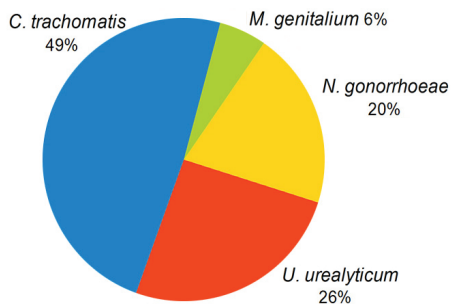

Figure 3. Distribution of sexually transmitted disease agents in positive samples

Age distribution of all patients and positive patients respectively are shown in Figure 1. 16 out of 21 patients (76.19%) in the 20 to 39 years age group were positive, by far the highest percentage of all age groups.

The 35 DNA samples collected from 28 patients, including 7 double infections and one triple infection, were subjected to statistical analysis. If, according to the hypothesis, all six pathogens may occur in the investigated cases, the probability of them not to develop one infection is p = 0.166. The mathematical expectation of the presence of species is M (n) = 5.833. The criterion (χ^2) employed to establish whether there was a significant deviation of STD pathogen species occurrence compared to the mathematical expectation of their occurrence is expressed as,

$$\chi^2 = \frac{(F - n \cdot p)^2}{n \cdot p}$$

where F is the frequency of occurrence, while n sums the presence of pathogen species. Test results are shown in Table II.

Discussion

Precision and accuracy of DNA extraction

For molecular diagnosis of *N. gonorrhoeae* and *C. trachomatis*, CDC recommends the urine samples (16). Riemersma et al. (17) and Nelson et al. (18, 19) have used FVU samples to reveal the presence of microorganisms in the urethra. Dong et al. (20) collected paired urine and swab specimens from 32 men and compared

Table II. χ2 test calculation results

Species	***C. trachomatis***	***N. gonorrhoeae***	***U. urealyticum***	***M. hominis***	***M. genitalium***	***T. vaginalis***	**Total**
Simple infection	12 (10.68)	3 (4.4)	5 (5.65)	0	2 (1.25)	0	22
Double infection	4 (4.85)	3 (2)	3 (2.57)	0	0 (0.57)	0	10
Triple infection	1 (1.45)	1 (0.6)	1 (0.77)	0	0 (0.17)	0	3
TOTAL	17	7	9	0	2	0	**35**

the results using multiplex 16S rRNA gene PCR. They demonstrated that the microbial load in male FVU and urethral swab specimens is nearly identical, independent of STD or other inflammation (10). In line with these recommendations, we collected FVU samples from all patients. When a middle jet is collected there is a possibility that STD pathogens are removed and an actual infection, if not massive, could go unnoticed

Obtaining DNA from urine is a rather difficult process. For urine samples, using fresh FVU specimens, the assay's sensitivity may decrease if samples are repeatedly frozen/thawed or stored for long periods of time because nucleic acids can easily be degraded. To avoid sample alteration, specimens were immediately transported to the laboratory. To avoid freezing, DNA extraction was carried out in the same week samples were collected - during which time samples were stored at 2-8 °C.

One of the limitations regarding the method employed involved the quantity and quality of the DNA obtained from urine samples. It was observed that the urine quantities needed depended on the microbial load. The amount of urine required was therefore accordingly adjusted: for high turbidity samples amounts of 1 mL were sufficient, while low turbidity samples required larger amounts (up to 12 mL).

The A260/280 and A260/230 ratios determined via spectrophotometric measurements were used to convert optical density values into DNA concentrations, thus assessing DNA purity. All high turbidity samples presented low A260/A280 ratios, implying massive proteinuria. Further DNA purifications were needed for these samples to comply with protocol requirements needed for the PCR reaction.

In order to achieve appropriate results, adequate DNA concentration and purity are needed to facilitate PCR amplification. The method we used gave satisfactory results in this regard, compensating the length of this laborious procedure.

Identification of STD pathogen agents via multiplex PCR

The results we obtained in the simultaneous detection of the six STD pathogens are satisfactory as the PCR amplification reaction occurred (the presence of internal control lanes being observed in all samples, see Figure 2). The employed method proved to be accurate, as demonstrated by the two (negative and positive) controls: while the negative control showed there was no contamination on any band, the positive control exhibited bands corresponding to the six DNA fragments of known molecular weight in the STD6 ACE marker that we used to determine the targeted products' size (Figure 2).

In recent decades bacterial cultures have been widely used for detecting STD pathogens. As mentioned, these pathogens (*U. urealyticum*, *M. hominis* and *T. vaginalis* in particular) are difficult to be cultured because they present a slow-growing rate, they need strict nutrient requirements and specific culture media that are not widely available. (13). As for *M. genitalium*, many researchers would rather turn to serology and PCR methods if needing to document an infection caused by this bacterium (21). According to some authors, *M. genitalium* requires a special medium and incubation for up to 8 weeks in order to be detected (15). The utility of the multiplex PCR was proven, as we identified *M. genitalium* in a symptomatic patient for whom previous tests using various culture media gave no positive results.

The method used in our study was tested and evaluated by comparison with the classical method and other PCR methods in previous studies (15, 22) and the results demonstrated that multiplex PCR has a high sensitivity, specific-

ity and rapidity in the simultaneous detection of multiple STDs and can be used for routine analysis in detecting these six pathogens. Potential limitations in using the multiplex method include the stochastic fluctuation in the interactions of PCR reagents particularly in the early cycles (PCR drift) or the competitive inhibition by the PCR selection which inherently favours the amplification of certain templates due to the relative target concentrations (22).

However, this multiplex PCR technique is clearly more convenient and effective. Other studies have also found that RT-PCR technique is more sensitive and accurate in detecting DNA compared to the classical methods (11, 12, 23). As we know, STD pathogens are frequently associated and are simultaneously transmitted from infected persons to their sexual partners. Using this method, the most frequent six pathogens causing STDs can be simultaneously detected in the same sample. Moreover, in our study we detected six cases of multiple infections using the same DNA (Table I). These findings are consistent with literature data (15, 24, 25).

Distribution of STD pathogens in the study group

Our results highlighted the raised frequency of STDs (over 50%) in the study group. Regarding the STD-symptomatic patients enrolled in the study, most cases were detected in the 20-29 and 30-39 years age groups. The number of patients over 40 years old is significantly lower (Figure 1). Given that 76.19% of the 20-29 years age group and 43.47% of the 30-39 years age group were found to be positive, one can say there is a high frequency of STDs in the younger population.

The predominance of the three most common STD-causing pathogens (*C. trachomatis, N. gonorrhoeae, U. urealyticum*) is consistent with data from literature (26, 27). The most frequently isolated STD pathogen was *C. trachomatis* (frequency of 32.69%, see Table I). Other studies also reported *C. trachomatis* as the most prevalent bacterial STD (26, 28). Next two in the prevalence scale, *U. urealyticum* and *N. gonorrhoeae* (frequency of 17.31% and 13.46%), were found in relatively equal proportions, a finding also consistent with prevalence studies conducted in other countries (27, 29).

In our study six cases of mixed infections with two and even three STD pathogens were detected. These multiple infections involved 11.54% of the patients, a fairly large proportion. The association of various pathogens that cause STDs is quite common due to biological factors, such as a common route of transmission, the partners' sexual behavior and the associated flora (25).

Mixed infections can be a cause of recurrent urethritis and cervicitis and this method is able to determine in the DNA extracted from a single urine sample the presence of several pathogens exhibiting similar signs and symptoms. By using this method, our study highlighted the raised prevalence of such infections and the need for multiple testing even if only one pathogen agent is presumably involved.

Interpretation of statistical results

In our case the χ^2 calculated value is 3.065. Taking into consideration that in our study the number of degrees of freedom expressed as (number of rows - 1) × (number of columns - 1) was 10, the χ^2 calculated value corresponds to a significance level α ranging from 0.990 to 0.975. In these circumstances the null hypothesis cannot be rejected. We conclude that the difference is statistically significant, not admitting that differences between the actual and expected frequency of STD pathogens is coincidental.

In conclusion, STD frequency in the study group was 53.84%. The most common STD pathogens detected were *C. trachomatis*, *U. urealyticum* and *N. gonorrhoeae*, the highest fre-

quency being observed in the 20-29 years age group.

Statistical analysis provides arguments to support the need for further investigating the causes leading to the large number of infections with the most frequent STD pathogens. The multiplex PCR method we used is rapid and very sensitive for the simultaneous diagnosis of the most common six STDs whose frequency is higher in our country, especially in the younger population.

Wide scale application of the system based on simultaneous detection of these six STD inducing pathogens may facilitate diagnosis in multiple infections. We propose this technique to be used in population screening on STD distribution.

Acknowledgements

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Abbreviations

STD - sexually transmitted disease
WHO - World Health Organization
CDC - Centers for Disease Control and Prevention
PCR - polymerase chain reaction
FVU - first void urine
TBE - Tris-borate 0.1 mM EDTA
IVD - In Vitro Diagnostic

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