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Original Article

Assessment of STI screening in Romania using a multiplex PCR technique

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Abstract

Introduction: Most sexually transmitted infections (STIs) are curable, but inappropriate treatment can lead to serious complications. The importance of setting up STI screening programs has been highlighted in various studies, the absence of such national programs accounting for the lack of STI statistics in Romania. The purpose of our study was to evaluate multiplex PCR as a screening method for the most common 6 STIs and establish their frequency in a group of symptomatic and asymptomatic patients. We aimed to highlight STI associations and correlations between STI pathogens and symptomatology, demographic status, antecedents or sexual partners.

Methodology: A total of 249 patients, both symptomatic and asymptomatic, were included in this study. *Chlamydia trachomatis* (CT), *Neisseia gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), *Ureaplasma urealyticum* (UU), *Mycoplasma hominis* (MH) and *Mycoplasma genitalium* (MG) were all identified in urine samples via multiplex Polymerase Chain Reaction (PCR). The SPSS IBM program was employed for statistical analysis.

Results: 32.12% of the patients were found positive, some presenting multiple infections. The results are representative for the Romanian male population. 107 STI pathogens were identified, most frequent being CT, UU and NG. Several statistical correlations between patient characteristics and the presence of STIs have been demonstrated.

Conclusions: The results suggest that multiplex PCR meets all the prerequisites for a screening method, allowing the use of multiple specimens and enabling simultaneous detection of multiple pathogens in a short period of time. STI identification via multiplex PCR proved to be an effective method for quantifying their frequency in Romania.

Key words: STI; multiplex PCR; screening.

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Introduction

Sexually transmitted infections (STI) are regarded as common diseases and the increasing number of asymptomatic forms lead to their under-diagnosis and wide dissemination, undiagnosed patients acting as source for further contamination of other individuals. In the absence of a widespread quick, cheap, sensitive and specific diagnostic method, a constantly increasing number of patients are not (or under) diagnosed and actively contribute to the dissemination of these diseases [1].

Without adequate treatments, available for most STIs, various short-, medium- or long-term complications may occur, including cervicitis, urethritis, premature birth, pelvic inflammatory disease (PID), increased susceptibility to human immunodeficiency virus, infertility or endometriosis [2,3].

Although any sexually active individual is at risk of developing a STI at some point, certain factors definitely increase the risk of a sexual infection: multiple or frequently changed intercourse partners, prostitution, cervical ectopia, lack of condom use, delay of medical consultation, lack of therapeutic compliance, low socio-economic and educational status or failure to have all sexual partners tested and committed to treatment [4-6]. Age, gender, menstrual cycle, pregnancy and use of contraceptives also need to be taken into consideration [7]. The young age (under 25) at the first sexual intercourse, the number of recent sexual partners, recent partner changes, a history of STI, alcohol or drug use are also contributing factors for developing STIs [8].

Currently over 30 bacteria, viruses and parasites are known to be transmitted through sexual (vaginal, oral and anal) contact. Many of these are acquired by the newborns at birth or during mother's pregnancy. According to a report from the Centers for Disease Control and Prevention (CDC), 1.7 million cases of *Chlamydia trachomatis* infections have been reported in 2017 in the USA alone, a 22% increase compared to 2013. More alarming were infections with *Neisseia gonorrhoeae*, a number of 555,608 new cases in 2017 reflecting a 67% increase over the same interval [9]. While reporting *C. trachomatis* and *N. gonorrhoeae* infections is mandatory in the USA, a lack of national statistics regarding STI frequency in Romania is due to the absence of national screening programs. Also, a relatively high percentage of young people with risky sexual behaviors and insufficient knowledge of the STI consequences are major concerns in our country [10].

Setting up screening programs for STIs is of paramount importance as various studies report a significant reduction in the medium and long term sequelae caused by these diseases. A randomized clinical trial in the USA reported that screening of women at high risk for *C. trachomatis* infection has reduced the incidence of PID from 28 to 13 cases per 1000 women per year [11]. STI screening can considerably reduce treatment costs and the risk of transmission to sexual partners, indirectly leading to a decrease in the occurrence of new antibiotic-resistant strains.

A wide variety of laboratory methods can currently identify STIs, from cell cultures to molecular testing ones [12]. The former has for a long time been considered the most specific STI diagnostic method, but the lengthy procedure and specialized equipment/personnel required led to the development of various culture-independent diagnostic techniques [11]. Another disadvantage of the cell cultures is that only viable bacteria can be identified, whereas nucleic acid amplification tests (NAATs) allow both viable and non-viable bacteria identification [13]. However, NAATs' main advantage over all other tests resides in its increased sensitivity and specificity [14]. Therefore, the Polymerase Chain Reaction (PCR) is a safe and rapid method that can process a wide range of specimens, including urine, vaginal/urethral secretions or sperm.

Considering the inconveniences of cell cultures, a syndromic management of STIs is rather frequent in Romania. This prompted us to evaluate the multiplex PCR for screening the most common 6 STIs in a group of patients. We aimed to determine the frequency of the 6 STIs among symptomatic and asymptomatic patients, to establish STI associations and correlations between STI pathogens and symptomatology, demographic status, antecedents or sexual partners.

Methodology

Participants

This transversal observational study was carried out between January 2014 and March 2017 at the Dermatology Clinic within the Cluj County Clinical Emergency Hospital in Cluj-Napoca, Romania, in collaboration with the Cell and Molecular Biology Department of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca. A total of 249 participants aged 17 to 75, both symptomatic and asymptomatic, were included in this study. The patients were recruited in the Urology, Dermatology and Infectious Diseases Departments, in a multidisciplinary approach. All participants provided information on age, gender, origin background, sexually active/inactive status, symptomatic status, marital status, socio-economic status, history of STI, presence/absence of symptoms in the sexual partners, previous STI test results. All participants gave informed consent to be included in the study and approval was obtained from the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca (no. 146/15.04.2014).

Criteria for inclusion /exclusion

The group of symptomatic patients included subjects accusing characteristic STI symptoms, namely vaginal/urethral secretions, dysuria, hematuria, pollakiuria, lower abdominal pain, sometimes combined with fever.

Asymptomatic patients included subjects not experiencing such symptoms whose reasoning for testing requests were assessment of their health status, a symptomatic STI sexual partner or engagement in unprotected sexual practices.

Patients receiving antibiotic treatment in the two weeks prior to testing were excluded from the study.

Polymerase chain reaction (PCR)

Considering invasiveness a key issue, our DNA choice was urine sampling. All 249 subjects were given sterile recipients and requested to provide 30-50 mL of the first urine jet collected early in the morning following at least 4 hours of no urine output. DNA was extracted from 150 µL of concentrated urine with an Epicentre MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE Biotechnologies, Madison, Wi, USA). DNA concentration and purity

were determined with an OD600 NanoPhotometer (Implen, Munchen, Germany) and adjusted when needed.

A Seeplex STD6 ACE Detection kit (Seegene, Seoul, Korea) containing primer pairs for *C. trachomatis* (CT), *N. gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), *Ureaplasma urealyticum* (UU), *Mycoplasma hominis* (MH) and *Mycoplasma genitalium* (MG) was employed in a multiplex PCR. This kit „can be used for IVD (*In Vitro* Diagnostic) purposes in EU", as mentioned in the user manual, having been tested by the manufacturers in regard to specificity, sensitivity, reproducibility, clinical performances and quality control. The amplicons were subjected to agarose gel electrophoresis (stained with 2% ethidium bromide) and examined using a UV trans-illuminator. Manufacturers' recommendations were strictly followed throughout the procedures.

Statistical analysis

IBM SPSS Statistics 23.0 was used to analyze both qualitative (nominal variables with dichotomous scales) and quantitative (continuous variables) data. Considering that data distribution was non-parametric, the statistical tests used were the Spearman's rank-order correlations, the Chi Square Test (χ^2) and the Fischer's Exact Test. Frequencies for each dichotomous variable were calculated, as well as the mean and standard deviation of the continuous variables. Significance thresholds were set to 5%. Graphic representations were generated in Microsoft Excel.

Results

The general characteristics of the study group are presented in Table 1. The average age of the 249 subjects (predominantly males) was 32.63 ± 9.5 years. Distribution by age groups highlighted that most patients were in the 21-30 years of age range, closely followed by the 31-40 years group. The studied population was predominantly unmarried, symptomatic and without a personal STI history.

Table 1. Patient general characteristics.

Characteristics	Cases n (%)
Age (years)	
< 20	4 (2)
21-30	117 (48)
31-40	94 (38)
41-50	18 (7)
51-60	8 (3)
61-70	3 (1)
>70	3 (1)
Gender	
Males	207 (83)
Females	42 (17)
Marital status	
Married	85 (34)
Single	165 (66)
STI antecedents	
Present	57 (23)
Absent	192 (77)
Clinical symptoms	
Present	181 (73)
Absent	68 (27)

Eighty (32.12%) of the 249 patients were found positive, some of them presenting multiple infections. Overall, 107 STI pathogens were identified in the samples, the most common being CT, which was present in half of the positive patients. The next most frequent were UU, identified in 22 cases, and NG, present in 18 cases. MG and MH were identified in 7 and 5 patients, respectively, while one patient presented a TV infection.

Regarding the pathogen distribution by age, the most infections involved patients aged 21 to 30 years, while the lowest number was observed in those over 40 years of age. The most cases of CT and NG were noted between 21-30 years, while UU infections were equally divided between the 21-30 years and 31-40 years' groups.

Table 2 highlights the patients' distribution according to gender and pathogen agents. The most positive results involved men (73 cases, 35% of total male number). In contrast, only 7 women (20%) were

Table 2. Distribution of patients according to gender and pathogen.

	Males (% of total males number)	Females (% of total females number)	Chi square (χ^2) $p < 0.05$	Fischer's exact test $p < 0.05$
CT	50 (24.2)	4 (9.5)	0.036	0.040
MG	7 (3.4)	0	0.227	0.606
MH	3 (1.4)	2 (4.8)	0.163	0.199
NG	17 (8.2)	1 (2.4)	0.183	0.324
TV	0	1 (2.4)	0.026	0.169
UU	20 (9.7)	2 (4.8)	0.308	0.388

CT: *Chlamydia trachomatis*, MG: *Mycoplasma genitalium*, MH: *Mycoplasma hominis*, NG: *Neisseria gonorrhoeae*, TV: *Trichomonas vaginalis*, UU: *Ureaplasma urealyticum.*

Table 3. Pathogen distribution according to STI antecedents.

	Antecedents		Chi square (χ^2) $p < 0.05$	Fischer's exact test $p < 0.05$
	Present	Absent		
CT	13	41	0.815	0.855
MG	1	6	0.583	1.00
MH	1	4	0.876	1.00
NG	6	12	0.274	0.259
TV	0	1	0.585	1.00
UU	6	16	0.608	0.600

CT: *Chlamydia trachomatis*, MG: *Mycoplasma genitalium*, MH: *Mycoplasma hominis*, NG: *Neisseia gonorrhoeae*, TV: *Trichomonas vaginalis*, UU: *Ureaplasma urealyticum.*

found positive. While nearly one-fourth of the men were diagnosed with a CT infection, no TV infection was observed in their case. CT infections were identified in four of the seven positive women, in whom no MG infection was found.

STIs were more common among unmarried patients, except for the MH infection which was more frequent among married couples.

Table 3 illustrates that most pathogens were identified in patients who did not have a personal history of STI.

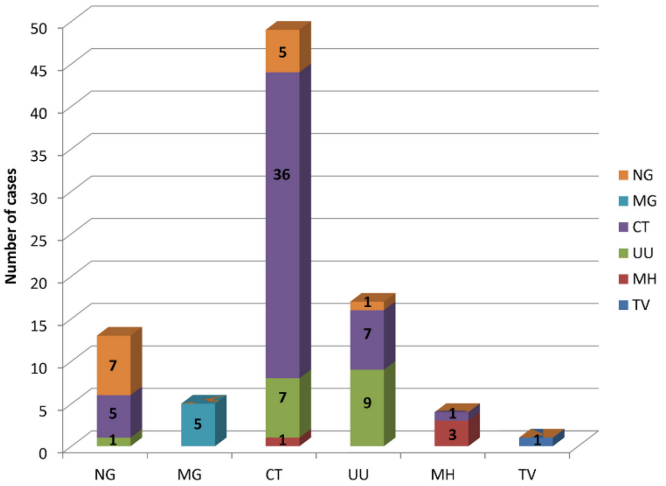
PCR analyses were performed for 176 patients previously examined, while for 73 patients the procedure served as STI diagnostic method. 181 patients in the analyzed group presented STI symptoms, while 68 patients declared they experienced no symptoms. STIs were identified via PCR in 49.58% of the symptomatic patients and in 41.66% of the asymptomatic ones. 20.4% of the symptomatic patients and 25% of the asymptomatic patients presented CT infections. NG was more frequent in symptomatic patients (7.7%) compared with asymptomatic ones (5.9%). None of the patients diagnosed with MH infection was asymptomatic (see Table 4). Most sexual partners of the patients enrolled in the study did not experience typical STI symptoms.

Several associations among pathogens were found in this study, as presented in Figure 1. A triple association involving UU, CT and NG was found in 3 patients. A quadruple association between UU, CT, NG and MG was found in 1 patient. Five associated bacteria, namely UU, CT, NG, MG and MH were identified in another case.

Discussion

We employed the multiplex PCR method with satisfactory results, given the large number of positive patients and of pathogens identified. As time is a crucial factor in the diagnosis and treatment of STI, a global trend to replace cultures as a diagnostic method is observed considering that up to 7 days are needed to

Figure 1. Associations of STI pathogens.



CT: *Chlamydia trachomatis*, MG: *Mycoplasma genitalium*, MH: *Mycoplasma hominis*, NG: *Neisseia gonorrhoeae*, TV: *Trichomonas vaginalis*, UU: *Ureaplasma urealyticum.*

Table 4. STI distribution according to symptoms.

	STI symptoms		Chi square (χ^2) $p < 0.05$	Fischer's exact test $p < 0.05$
	Present	Absent		
CT	17	37	0.437	0.491
MG	2	5	0.939	1.00
MH	0	5	0.166	0.327
NG	4	14	0.615	0.786
TV	0	1	0.539	1.00
UU	5	17	0.513	0.803

CT: *Chlamydia trachomatis*, MG: *Mycoplasma genitalium*, MH: *Mycoplasma hominis*, NG: *Neisseia gonorrhoeae*, TV: *Trichomonas vaginalis*, UU: *Ureaplasma urealyticum.*

cultivate certain bacteria, while a NAAT via PCR can be completed in several hours. As for the DNA specimen we selected for the PCR analysis, urine, was recommended by its non-invasive method of collection that doesn't require the presence of qualified staff (self-collection). In order to avoid cross-contamination, the patients were offered in-advance details regarding the sample collecting protocol and were asked to confirm that the protocol was followed and the provided recipients were used.

We selected an age-representative sample for this study based on the CDC's report that about half of the newly diagnosed STIs are observed in young adults aged 15-24 [15], as well as on the results of our previous study [16]. The mean age of the population sample was 32.63 years, 48% of patients being in the 21-30 years range and 38% in the 31-40 years range. The inclusion of subjects under the age of 18 was limited as it required parental agreement. A higher frequency of positive results was observed in the 21-30 years range, as the negative correlation (Spearman r_s = -0.13*) observed for the age groups over 30 years (age being the continuous variable) confirms that the positive results are more rarely encountered over the age of 30.

Most of the patients enrolled in the study were men (83%), explainable since STIs symptoms are more frequently observed in this category. Males also provided most positive results (73), a statistically significant score (Chi Square χ^2 (1, N = 249) = 5.539, p = 0.019; Fisher Exact Score p = 0.019) being noted. Most women with CT infection were asymptomatic, avoidance of medical examinations leading to the development of long-term complications such as PID and infertility. In the absence of symptoms, women serve as STI dissemination pool. The results of the Chi square test χ^2 (1, N = 249) = 4.4, p = 0.036 and the Fisher test p = 0.040 confirmed an association between CT and the patients sex.

Although a personal history of STI is one of the most important risk factors [17], only a quarter (23%) of the patients included in the study claimed they had been previously diagnosed with this pathology. Most of the highlighted STIs were identified in subjects without a personal history, due to their higher ratio in the present study. No statistically significant associations between a personal history of STI and the responsible pathogenic agents (p > 0.05, Table 3) or between the detected STI frequency and the frequency of the previous analyzes (p > 0.05) were found, based on the correlation tests we performed. This can be due to the fact that the PCR has a higher sensitivity and accuracy than other methods of analysis.

Almost three quarters (73%) of our patients were symptomatic, suggesting their low interest regarding their own state of health. No statistical significance was established between the frequency of the positive results in symptomatic patients compared to asymptomatic ones (Chi Square χ^2 (1, N = 249) = 0.317, p = 0.574; Fisher's exact test p = 0.649), denoting that these infections may sometimes be asymptomatic and, due to the large number of positive samples detected in the asymptomatic patients group, a screening in the general population would be needed in Romania.

In regard to the sexual partners' symptoms, CT has been reported by 8 partners, while other 46 presented no symptoms. The lack of gonorrhea symptoms in any of the sex partners of the patients included in this study statistically correlated with NG infections (Chi Square χ^2 (1, N = 249) = 3.937, p = 0.036 and Test Exactly Fisher's p = 0.049).

CT was the most common STI diagnosis, providing half of the positive results identified via PCR, in accordance with literature data and the conclusions of our previous studies [16,18-20]. Considering the very high number of CT infections in our patients and other recent reports in our country [21], it is obvious that Romania lacks an adequate system of reporting and quantifying the exact number of infections with this pathogen. In the present study, CT was the most common STI agent diagnosed in males (24.2% vs. 9.5% in women). Our finding comes in agreement with a previously one conducted in Norway [22]. The association between CT and males was statistically significant (Chi square χ^2 (1, N = 249) = 4.4, p = 0.036 and Fisher exact test p = 0.040). CT was relatively evenly distributed among asymptomatic (25%) and symptomatic (20.4%) patients. 25.6% of all unmarried persons included in the study were diagnosed with CT. While CT was the most common infection among young subjects in the 21-30 years range (32 cases highlighted), it was very poorly identified in those over 40 years.

The second most frequent infectious agent in the tested samples was UU (21%), most of the 22 cases being identified in patients aged 21 to 40 years. This infection was more often found in symptomatic patients, as reported in another study [23]. Men were more frequently affected by UU infections than women, a result that overlaps data from a Turkish study where UU was identified in 28.3% of symptomatic men [24]. UU under-diagnosis could result from the absence of a method to easily identify this bacterium, knowing that its development on culture media is very difficult. Another cause of sub-diagnosis is the syndromic

management of STIs, antibiotic treatment being often recommended without diagnosing the etiological agent.

NG infection represents a major therapeutic problem worldwide, but its frequency in Romania is unknown in the absence of a national STI reporting program. According to CDC statistics, gonorrhea is the second-rated STI in the USA after the CT infection, and the number of newly diagnosed cases has increased since 2013 [25]. Gonorrhea was ranked the third most frequent in our study, being identified in 18 (17%) of the 249 patients included in the research, best represented in the 21-30 years range. NG was more often observed in symptomatic patients ($p = 0.615$). A statistically significant conclusion (Chi Square χ^2 (1, N = 249) = 3.937, $p = 0.036$; Fisher Exact Test $p = 0.049$) was that the sexual partners of the patients diagnosed with gonorrhea did not accuse characteristic symptoms. 14 of the subjects diagnosed with NG infection were previously examined, but persistence of the symptoms determined them to request the PCR analysis.

MG was found in 8% of the subjects, all of them males aged 21 to 40 years. None of the women enrolled in the study was infected with MG. The fact that MG infection is more common in males was also stated in a previous study [22].

MH infection was identified in 5% of our subjects, symptomatic patients in the 21-50 years range ($p = 0.166$). The reduced number of MH infections we found may be due to the small number of women included. In a study that included 200 women, MH was the most common STI, being identified in 135 cases [26].

The only case of TV infection in this study was found in a woman in the 21-30 years age range, consistent with literature data [27,28]. The small number of trichomoniasis may not necessarily be due to the reduced number of female samples, since in the previously cited study, including 200 women, TV was also identified only in one case [26]. Similarly, a recent study conducted in the USA in 2013-2016 exhibited a 1.3% prevalence of urine TV in a population aged 14-59 years [29]. This suggests that in the recent years the prevalence of this STI has dropped considerably.

It is documented that some of these STI agents may be present as normal flora in a large percentage of the sexually active individuals. We have actually determined the presence of all the STI agents as we consider their detection in the commensal flora is important since they can be transmitted to uninfected persons who may consequently develop infections.

Regarding the associations among STI pathogens, the most common we found, between CT and UU (7 cases), was statistically significant (Chi Square χ^2 (1, N = 249) = 15.241, $p < 0.001$; Fisher's Exact Test $p < 0.001$). This association was more frequent in our study than in the one conducted by Jensen et al., which reported 4 cases in a batch of 665 people [22], but less often than in that of Kim et al., who identified the CT-UU association in 24 cases from 463 subjects [27].

As the frequent association between CT and NG is well documented, diagnosis of gonorrhea is recommended when CT infections are identified. In the present study, the association between CT and NG, detected in 5 cases, was statistically significant (Chi Square χ^2 (1, N = 249) = 13.105, $p < 0.001$; Fisher Exact Test $p = 0.001$). Another study identified 15 CT-NG associations in 436 male patients [27].

The association of UU with NG was identified in one case, quite similar to a previously cite study where this association was identified in 2.6% of the cases [27]. No associations were found for MG and TV, which have only been identified as singular infections.

The relatively small number of patients included and the high male percentage are limitations of this study that led to the absence of statistically significant results regarding the STIs distribution according to gender. Because most of the patients were men, it can be said that results are representative for the Romanian male population. However, as the STIs frequency (in descending order CT, UU, NG, MG, MH and TV) identified here by us coincides with that reported in a study conducted on 436 men [27], we consider that these data may be extended to the general population.

Our results indicate that Seeplex STD6 ACE Detection kit can be successfully used for STI screening in the population. There is also a new version of the Seegene assay that could be used for such screening. One feature of the present study is the identification of double, triple, quadruple and even quintuple associations. Since the association of several pathogens is a frequent cause of treatment failure, the PCR method demonstrated its usefulness as it allowed simultaneous identification of multiple pathogens in the same specimen.

Conclusion

Multiplex PCR is an adequate method for the diagnosis of STIs, allowing the use of multiple specimens and simultaneous detection of multiple pathogens. Its quickness, high sensitivity and specificity are advantages that recommend it as a screening method for large populations.

The identification of STIs via multiplex PCR proved to be an effective method for quantifying their frequency in Romania. Considering the very high

number of pathogens in our samples and other recent reports in regard to our country, it is obvious that Romania lacks an adequate system of reporting and quantifying the exact number of infections with STI agents.

From our study, we were able to extract useful information about STIs in Romania. The results are representative for the Romanian male population. Our findings indicate that STIs are more common among men and subjects aged 21-30 years. The most common STI identified in this study was the CT infection, the next most frequent being UU and NG infections. MH and MG were rare in the studied population, while TV was identified in only one case. We found multiple STI pathogen associations that could result in failure of antibiotic treatment.

Our results are consistent with literature data, suggesting that PCR multiplex presents all the features required for a screening method. The scarceness of data on STIs frequency in Romania confers originality to the present study.

Authors' contributions

All authors contributed equally to this work.

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