Evaluation of Symptomatic Patients with Resistant Discharge

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ABSTRACT The aim of this study was to detect the presence of *Chlamydia trachomatis, Neisseria (N.) gonorrhoeae, Mycoplasma (M.) hominis, M. genitalium, Ureaplasma (U.) urealyticum*, and *Trichomonas (T.) vaginalis* in patients with resistant discharge. The study also evaluated the concordance of the diagnostic tests. Samples from 156 patients were tested by direct microscopy and culture for *T. vaginalis* and Mycoplasma IES for *M. hominis* and *U. urealyticum*. Multiplex Polymerase Chain Reaction (PCR) was used to determine the presence of six agents. Statistical analyses were performed using the SPSS program. Out of 156 patients, 38 had positive result for the agents tested. Of these 38 patients, 28 (73.7%) had single agent positivity and 10 (26.3%) had multiple agent positivity. The detection rate of *U. urealyticum*, *M. hominis*, *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, *M. genitalium* specifically was 10.3%, 9.6%, 6.4%, 3.2%, 2.6%, 0.6% respectively. *N. gonorrhoeae* and *U. urealyticum* were the most common in male patients, while *M. hominis* and *U. urealyticum* were mostly found in female patients. Different methods used for detecting *T. vaginalis* were compared to find that interrater reliability was perfect for culture-direct microscopy (κ:0.85; $P<0.001$) and also for culture-PCR (κ:0.89; $P<0.001$). The interrater reliability was moderate (κ:0.53; $P<0.001$) for PCR-Mycoplasma IES test for *M. hominis* and fair (κ:0.21; $P<0.007$) for *U. urealyticum*. *U. urealyticum* and *M. hominis* were among the most commonly found sexually transmitted infections (STI) agents in patients with resistant discharge. Multiple agent positivity was high and should be kept in mind in every STI case.

KEY WORDS: STI, *Ureaplasma urealyticum*, *Mycoplasma hominis*, diagnostic methods

INTRODUCTION

Sexually transmitted infections (STI) create social and economic public health problems throughout the World (1). WHO statistics show that about 357 million new cases of STIs occur worldwide in adults between the ages of 15 and 49 years. In both sexes, up to 70% of infected persons remain asymptomatic for a period of time (1,2).

A large number of detection methods and different types of patient specimen types in which STIs can be determined are available for clinical laboratory use (3). Recent reports showing that an STI often involve a group of variable pathogens have prompted a search for methods that identify multiple pathogens in a single sample (4). The Seeplex STD ACE (auto-capillary

electrophoresis) Detection Assay is a multiplex Polymerase Chain Reaction (PCR) based semi-automated detection system that employs 6 pairs of dual priming oligonucleotide (DPO) primers targeted to the unique genes of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *U. urealyticum*, *M. hominis*, and *T. vaginalis*. Furthermore, the assay was reported to be a cost-effective and fast diagnostic tool with high sensitivity and specificity for the simultaneous detection of multiple STI pathogens (5).

The prevalence of STI involving *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *U. urealyticum*, *M. hominis*, and *T. vaginalis* in the Turkish population is not fully investigated due to the lack of standardized diagnostic methods and problems in the notification system. The objective of the present study was to investigate the presence of *C. trachomatis, N. gonorrhoeae, M. genitalium, U. urealyticum, M. hominis*, and *T. vaginalis* in symptomatic male and female patients and additionally to evaluate the concordance of multiple diagnostic tests for *U. urealyticum*, *M. hominis*, and *T. vaginalis*.

PATIENTS AND METHODS

Patient cohort: A total of 156 patients, 60 (38.5%) men and 96 (61.5%) women, admitted to the Public Health General Directorate, Microbiology Reference Laboratory in Ankara between May 2015 and February 2017 with vaginal and/or urethral discharge resistant to routine treatment, were enrolled in the study.

Sample collection: The cervical, vaginal, and urethral samples were obtained by sterile nylon non-aluminum swabs (Copan Flock Tecnologies, Italy). From each patient, a total of 3 samples (2 cervical and 1 vaginal for women and 3 urethral samples or urine sample for men) were obtained. The samples were inserted into the Universal Transport Medium (UTM, Copan Tecnologies, Italy) kit. Urine samples were sent in sterile cups. All the samples were sent to the laboratory at room temperature. Except for the ones which would be used for PCR test, all the samples were studied on the same day, while the samples for PCR were stored at -20 °C until analyzed.

Cervical samples: The first cervical sample was used for *M. hominis* and *U. urealyticum* testing, and the second one was used for multiplex PCR testing.

Urine sample: The first void urine sample was collected and was used for *T. vaginalis*, *M. hominis*, and *U. urealyticum* testing as well as for multiplex PCR testing.

Urethral samples: While collecting urethral samples, it was taken into consideration that the patients did not urinate within the last 2 hours. One of the urethral samples was used for *T. vaginalis* testing, the other one for *M. hominis* and *U. urealyticum* testing, and the third one for multiplex PCR testing.

Vaginal sample: The vaginal sample was used for *T. vaginalis* direct microscopy and culture testing.

For *T. vaginalis*, diagnosis samples were cultured on the Modified Diamond Medium (Hardy Diagnostics, USA) after a direct microscopic examination. Microscopic evaluation of the cultures was performed after three days, following daily observations. For the diagnosis of *M. hominis* and *U. urealyticum*, culture-based Mycoplasma IES was used. The DPO™-based multiplex PCR method was applied for all the patients for the detection of six agents.

Mycoplasma IES: The identification of *M. hominis* and *U. urealyticum* was performed with a commercially available Mycoplasma IES kit (Autobio Diagnostics, Belgium) which combines a selective culture broth with a strip. The test was performed according to the manufacturer's recommendations. Colony forming units of $\geq 10^4$/mL were considered a positive result for *M. hominis* and *U. urealyticum* infection.

Multiplex PCR Assay: Ribo Spin VRD kit (Gene All, Seoul, Korea) and Seeplex STD6 ACE Detection test (Seegene, Seoul, Korea) specifically designed primer sets for *C. trachomatis*, *N. gonorrhoeae*, *M. hominis*, *M. genitalium*, *U. urealyticum*, and *T. vaginalis* were used for DNA isolation and for PCR amplification respectively, according to the manufacturers recommendations. Amplification products were detected with the Screen Tape System (Lab 901 Limited, UK). Results were analyzed automatically with Screen Plex (Lab 901 Limited, UK) software.

Statistical Analysis: Statistical analyses were performed using SPSS software version 23.0 (SPSS Inc. Chicago). Descriptive analyses were given using number and percentage distributions, mean, and Standard Deviation. The concordance of the multiple methods used for diagnosis was evaluated by the Kappa test. The percentage of agreement between the results according to κ values were categorized as perfect (0.81-1.0), substantial (0.61-0.8), moderate (0.41-0.6), fair (0.21-0.4), slight (0-0.2) agreement (6). A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Out of the 156 patients who participated, 38 (24.4%) were positive and 118 (75.6%) were negative for all the pathogens tested. 57.9% (22/38) of the patients who tested positive were women and 42.1% (16/38) were men. Mean age was 36±13 (mean ± Standard Deviation). Group analysis by age group

showed that the highest percentage of infections was 26.3% (10/38) and was observed in the 20-24 age group, followed by the 35-39 and 40-44 years with a percentage of 18.4% (7/38) and 15.8% (6/38), respectively. When the identity of the specific pathogens in the study were investigated, 28 cases (73.7%) of 38 patients were positive for only one of the six agents for which diagnostic tests were performed, and 10 cases (26.3%) had positive results for more than one pathogen. The most commonly detected pathogen was *U. urealyticum* with a frequency of 10.3% (16/156), followed by *M. hominis* with 9.6% (15/156). The frequency of other agents were as follows: *N. gonorrhoeae*, 6.4% (10/156); *C. trachomatis,* 3.2% (5/156); *T. vaginalis,* 2.6% (4/156); *M. genitalium,* 0.6% (1/156) (Table 1).

N. gonorrhoeae and *U. urealyticum* were the most commonly detected pathogens in male patients, while *M. hominis* and *U. urealyticum* were most common in female patients (Table 2). Of the female patients, 81.81% (18/22) tested positive for only one pathogen, and 18.2% (4/22) had multiple pathogens. Of the male patients, 62.5% (10/16) were positive for only one pathogen, and 37.5% (6/16) had more than one pathogen.

Table 1. Descriptive characteristics of the study population

		n	%
Total samples		156	100.0
Positive samples		38	24.4
*Sex (n=38)			
	Female	22	57.9
	Male	16	42.1
*Age group (n=38)			
	15-19	1	2.6
	20-24	10	26.3
	25-29	3	7.9
	30-34	3	7.9
	35-39	7	18.4
	40-44	6	15.8
	45-49	3	7.9
	50-54	3	7.9
	55-59	0	0
	60-64	2	5.3
*Positive samples (n=38)			
	Single agent	28	73.7
	Multiple agent	10	26.3
**Agent (n=156)			
	U. urealyticum	16	10.3
	M. hominis	15	9.6
	N. gonorrhoeae	10	6.4
	C. trachomatis	5	3.2
	T. vaginalis	4	2.6
	M. genitalium	1	0.6

*Analysed by positive samples
**Analysed by positive agent number

T. vaginalis positivity was first assessed by the gold standard culture method. When the results of the culture method was compared with direct microscopy results, sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) were 75.0%, 100%, 100%, 99.3% and when compared with PCR the values were 100%, 99.3%, 80.0%, 100%, respectively. The interrater reliability, or concordance of the two tests compared, was perfect when the culture method was compared to direct microscopy (κ: 0.85; $P<0.001$), and when the culture method was compared to the PCR (κ: 0.89; $P<0.001$) results. *M. hominis* and *U. urealyticum* positivity were evaluated by PCR, and the results were compared with the culture-based Mycoplasma IES test. Sensitivity, specificity, PPV, and NPV were as follows; For *M. hominis*, 53.3%, 96.4%, 61.5%, 95.1%; for *U. urealyticum*, 37.5%, 87.9%, 26.1%, 92.5%. The interrater reliability was moderate (κ: 0.53; $P<0.001$) when PCR results were compared to the culture based Mycoplasma IES test for *M. hominis*, and fair (κ: 0.21; $P<0.007$) for *U. urealyticum* (Table 3). For the evaluation of *N. gonorrhoeae*, *C. trachomatis*, and *M. genitalium*, only the PCR method was used.

DISCUSSION

In the present study population, in both male and female patients, discharge was a common symptom. Abnormal vaginal discharge may be frequently observed due to vaginitis caused by *T. vaginalis*, *C. albicans*, or bacterial vaginosis. In some cases, vaginal discharge can be observed in cervicitis that is caused by *N. gonorrhoeae* and/or *C. trachomatis*. Urethral discharge is a common presentation of STI in male patients, and known pathogens include *N. gonorrhoeae*, *C. trachomatis*, and *M. genitalium* (3). In this study, the presence of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium*, and the flora elements *M. hominis*, *U. urealyticum* which are controversial as a pathogen were investigated.

The highest rates of STIs was observed among young individuals aged 20-24 years, with a rate of 26.3% (10/38), followed by those aged 35-39 and 40-44 years with a rate of 18.4% (7/38) and 15.8% (6/38), respectively. These results are consistent with the CDC 2016 STD Surveillance Report, where the 20 to 24 age group presented with the highest rate of STI (7).

Until now, few studies have simultaneously investigated the relative frequency of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *M. hominis*, *T. vaginalis*, and *U. urealyticum* in symptomatic cases in Turkey. In our study, the causative pathogens, *U. urealyticum*, *M. hominis*, *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, *and M. genitalium* were detected at a rate of 10.3%,

Table 2. Distribution of organisms according to gender

Organism	Female		Male		Total	
	N	%	N	%	N	%
Negatives	74	77.1	44	73.3	118	75.6
Single						
M. hominis	8	8.3	1	1.7	9	5.8
U. urealyticum	7	7.3	3	5	10	6.4
T. vaginalis	2	2.1	(-)	(-)	2	1.3
C. trachomatis	1	1	(-)	(-)	1	0.6
M. genitalium	(-)	(-)	1	1.7	1	0.6
N. gonorrhoeae	(-)	(-)	5	8.3	5	3.2
Multiple						
M. hominis, C. trachomatis	1	1	1	1.7	2	1.3
M. hominis, U. urealyticum	1	1	(-)	(-)	1	0.6
U. urealyticum, N. gonorrhoeae	(-)	(-)	3	5	3	1.9
C. trachomatis, N. gonorrhoeae	(-)	(-)	1	1.7	1	0.6
T. vaginalis, M. hominis	1	1	(-)	(-)	1	0.6
T. vaginalis, M. hominis, U. urealyticum	1	1	(-)	(-)	1	0.6
M. hominis, U. urealyticum, N. gonorrhoeae, C. trachomatis	(-)	(-)	1	1.7	1	0.6
Total	96	100	60	100	156	100

9.6%, 6.4%, 3.2%, 2.6%, and 0.6%, respectively. Consistent with our results, Leli *et al.* (8) reported that *M. genitalium* rates was 0.6%, and in the study by Ito *et al.* (9), *M. hominis* and *U. urealyticum* were detected in 10.7% and 8.9% of their test population, respectively. *M. hominis* was reported by Esen *et al.* (3) in 10.4% of patients tested, as well. Similarly, in the study by Sutton *et al.* (10), *T. vaginalis* was detected in 3.1%, and in a study by Masese *et al.* (11) *C. trachomatis* was detected in 3.6% of patients tested. Kahn *et al.* (12) reported that *N. gonorrhoeae* was detected in 6.4% of the patients. All these findings support our study. However, other clinical studies reported a total rate of 44.4% and 18.9% *M. hominis* and *U. urealyticum* infection, respectively, in Seoul (13) and *C. trachomatis* was found in 6.5% (14) in Estonian, 9% in Swedish (15), and 11% in Slovenian studies (16). In another study conducted in Turkey, the rate of *T. vaginalis* was found to be 8.1% (17). Differences in detection rates according to race and socioeconomic status have been reported, and differences by sex have also been suggested (4).

In this study, 118 (75.6%) of the patients were negative for all the agents investigated; however, from an epidemiological point of view, *U. urealyticum* and *M. hominis* were the most prevalent agents detected. *N. gonorrhoeae* and *U. urealyticum* were the most common in male patients, while *M. hominis* and *U. urealyticum* were mostly detected in female patients. In line with our results, a study in Korea also determined that the most prevalent pathogens

Table 3. Evaluation of different methods for the detecion of *T. vaginalis*, *M. hominis* and *U. urealyticum*

Agent		Culture — PCR			Culture — Direct Microscopy		
T. vaginalis		N	%	Kappa	N	%	Kappa
	Sensitivity	4/4	100.0		¾	75.0	
	Specificity	151/152	99.3	κ:0.89	152/152	100.0	κ: 0.85
	PPV*	4/5	80.0	P<0.001	3/3	100.0	P<0.001
	NPV**	151/151	100.0		152/153	99.3	
M. hominis							
	Sensitivity	8/15	53.3				
	Specificity	136/141	96.4	κ: 0.53			
	PPV	8/13	61.5	P<0.001			
	NPV	136/143	95.1				
U. urealyticum							
	Sensitivity	6/16	37.5				
	Specificity	123/140	87.9	κ: 0.21			
	PPV	6/23	26.1	P<0.001			
	NPV	123/133	92.5				

* PPV: Positive Predictive Value
**NPV: Negative Predictive Value

were *U. urealyticum* and *M. hominis* (18). Generally, *U. urealyticum* is not known as a clinical pathogen but it can be a causative agent of urethritis, especially in patients resistant to routine treatment. *M. hominis* also does not play a great role in the pathogenesis of urethritis, but it can be a major pathogen in immunocompromised patients (19). These findings show that *M. hominis* and *U. urealyticum* may be pathogenic agents in both sexes (20). Therefore, if these microorganisms are really pathogens, their early detection would be of great value. In the current study, finding *U. urealyticum* alone or together with other pathogens in 9.3% of female and 6.7% of male patients is an important result. High positivity of *U. urealyticum* in this group of symptomatic patients is striking and indicates that it can be considered as a cause of urethritis in both sexes. Furthermore, the presence of *U. urealyticum* was associated with urethral discharge in the study performed by Wetmore *et al.* (21). Although these findings suggest that *U. urealyticum* might have a role as a causative agent in lower genital tract infections, a number of risk factors, such as sexual behavior, number of partners, and recurrent urethritis should be considered. The limitations of this study include the lack of detailed demographic characteristics. We could not detect *T. vaginalis* in our male cases. This may be due to biological differences between the sexes. The iron-rich environment in the vagina provides favorable conditions for the growth of *T. vaginalis* and facilitates adherence and survival of the parasite in the genital region to the epithelium; while urine helps to remove it from the male genital tract, this mechanism is not expected to affect the clearance of vaginal secretions (22).

A very common phenomenon, the co-existence of various STI, has been attributed to several factors, including a common transmission route, the sexual behavior of the host, and the presence of resident flora (4). In the present study, 73.7% of all STI-positive cases were infected with one pathogen and 26.3% with multiple pathogens. Multiple agent positivity seems to be high and must be kept in mind in every case with chronic discharge. Single agent positivity was observed in 81.81% (18/22) of positive female patients, while multiple agent positivity was determined in 37.5% (6/16) of male patients. Co-infection among genital microorganisms has also been frequently reported in other studies (23).

Accurate and timely identification of STI agents is important for early treatment and prevention of disease transmission. For STI laboratory diagnosis, culturing swab specimens provides an accurate diagnosis; however, it has some disadvantages (24). Generally, culture is regarded as the gold standard method for the detection of *M. hominis*, *U. urealyticum*, and *T. vaginalis* in clinical samples. For genital mycoplasma and ureaplasma, commercially available diagnostic kits based on liquid broth cultures provide interesting alternatives to conventional culture (25). In our study, we used liquid-broth-based commercial culture Mycoplasma IES for *M. hominis* and *U. urealyticum* diagnosis. Sensitivity and specificity of the diagnostic methods used vary according to the gold standard or reference method used in the study. In a study by D'Inzeo *et al.* (25), sensitivities for the detection of *U. urealyticum* with Mycoplasma IES, Mycofast, and Mycoplasma IST 2 were 100%, 96.2%, and 95.3%, respectively, while those for *M. hominis* were of 92.8%, 92.8%, and 85.7%, respectively. As it is difficult to cultivate *Mycoplasma spp*. and *Ureaplasma spp*. conventional culture was not included as a reference method in our study. In Turkey, laboratories mostly use culture based kits instead of PCR, as it is generally cheaper. In this study, we compared the results of the two tests. When PCR was used as a reference method, we found the sensitivity of culture-based Mycoplasma IES test for *M. hominis* and *U. urealyticum* as 53.35% and 37.5% respectively, somewhat lower than that the one observed in the study by D'Inzeo *et al.* (25). Differences in sensitivity may be due to sampling and study design and also may be the result of the antimicrobial therapy received by the patients.

In order to strengthen microscopic detection, *T. vaginalis* diagnosis can also be made by cultivating the protozoon in various media. Modified Diamond is regarded as the most sensitive media since it provides an environment similar to vaginal epithelium due to its starch content. The culture method requires 3-7 days and is expensive when compared with direct microscopy, so the method is not used in routine laboratories, also due to the risk of contamination (26,27). Direct microscopic examination to identify *T. vaginalis* infections is the preferred method in routine use as it is fast and inexpensive; however, sensitivity and specificity of the test to detect motile parasites was 75% and 100% in our study, which is compatible with the sensitivity study (60% to 80%) by Saeed *et al.* (26). In various studies, it was determined that the specificity of the direct microscopy oscillated between 25% and 98.12% depending on the culture method, and the specificity was found to be 100% (28-30).

Co-infection with different pathogens is possible with STI; it is therefore essential to use different clinical samples and tests for accurate diagnosis, which is both time consuming and costly. Nowadays, kits used for simultaneous detection of multiple pathogens in a single sample are fast, easy, and are preferred by the laboratories for the diagnosis (24). The high

sensitivity and better specificity of Nucleic Acid Amplification Tests (NAAT) over other tests allows the use of various genital specimen types, including endocervical swabs, vaginal swabs, and urine samples for simultaneous detection of genital pathogens. Only viable bacteria can be quantified in bacterial culture, but NAATs such as PCR can quantify both viable and nonviable bacteria (2). The Seeplex STD6 ACE Detection assay is a multiplex PCR that is designed for the detection of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *U. urealyticum*, *M. hominis*, and *T. vaginalis* in a single sample. The test is cost effective, fast, and has high sensitivity and specificity, and was thus regarded as the reference method in *M. hominis* and *U. urealyticum* diagnosis in our study.

In the present study, the Seeplex STD6 ACE Detection assay was accepted as a practical and reliable method in the diagnosis of *T. vaginalis* when culture was considered as a gold standard method, with a sensitivity of 100% and specificity of 99.3%. The sensitivity and specificity of PCR varies between various studies, which is the reason for the different samples studied and primers used (27). In other studies, when compared with the culture method, it was determined that the sensitivity of PCR oscillated between 80% and 100%, and the specificity was between 97.9% and 99.3%, which is compatible with our results (28,29).

In our study, *U. urealyticum* and *M. hominis* were among the most prevalent STI agents in patients with chronic discharge. Multiple agent positivity was high and must be considered in every case with resistant discharge. Furthermore, the Seeplex STD6 ACE Detection assay was found to be a useful method, especially for the evaluation of symptomatic patients. Our results also indicate that direct microscopy and culture is a diagnostic tool that is compatible with the Seeplex STD6 ACE Detection assay for *T. vaginalis* diagnosis.

CONCLUSION

It is crucial to screen patients with discharge not only to identify symptomatic individuals for the diagnosis and management of their infections but also to limit transmission of the STI agents. *Ureaplasma urealyticum* and *Mycoplasma hominis* are among the most commonly detected STI agents in patients with resistant discharge. Multiple agent positivity is high and should be kept in mind in every STI case. Additional studies to more completely assess the significance of these findings are ongoing.

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