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Molecular diagnostic of *Ureaplasma urealyticum* presence and tetracycline resistance in urine samples

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Abstract

Sexually transmitted infections (STIs) are among the most common infections in Romania. Infection with Ureaplasma urelyticum is one of the major causes of STIs and can cause serious complications. Although tetracycline is the drug commonly used to treat infections caused by U. urealyticum, several studies indicate the emergence and rapid development of strains resistant to these antibiotics in the United States or Europe. Tetracycline resistance in bacteria is encoded by a number of different genetic determinants but in mycoplasmas the only tetracycline resistance determinant that has been reported is the tetM gene. Tetracycline resistance among Ureaplasma spp. is associated with the presence of the horizontally acquired tetM resistance gene. Our study on bacterial DNA aimed to determine the presence of tetracycline-resistant U. urealyticum strains, by identifying the presence of the tetM gene. We used first void urine samples from 622 STI-suspected subjects. DNA was extracted, purified and amplified via PCR for the simultaneous detection of 6 STIs. 68 patients were diagnosed with U. urealyticum. DNA obtained from these samples was amplified using the tetM gene and U. urealyticum - specific urease gene primers. The urease gene was amplified in all samples, confirming the presence of U. urealyticum. The tetM gene was amplified in 2 samples considered tetracycline-resistant strains. The study confirmed the presence of U. urealyticum strains resistant to tetracycline in Romania. The employed technique can produce quick results both for U. urealyticum detection and determination of its resistance to tetracycline using a single easy-to-collect biological sample.

Keywords: *PCR, Ureaplasma urelyticum, tetracycline-resistant, tetM gene, urease gene*

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Introduction

Sexually transmitted infections (STIs) are caused by a wide variety of bacteria, viruses and parasites intraspecifically transmitted mainly by vaginal, anal, or oral sexual contact. According to the statistics of the Centers for Disease Control and Prevention (CDC), more than 1 million STIs are daily acquired worldwide and the majority of STIs have no or only mild symptoms that may not be recognized as an STI (1). STI dissemination in the general population is an important health problem in Romania, amplified by the absence of a national screening program (2). A relatively high percentage of youngsters with risky sexual behavior and insufficient knowledge regarding STI consequences poses additional risks towards a population-wide disease spreading (3).

Ureaplasma urealyticum (belonging to *family Mycoplasmataceae*, *order Mycoplasmatales, class Mollicutes*) is among the most common STI-causing bacteria. Lack of a rigid cell wall prevents reactivity with Gram staining and do not respond to antibiotics targeting bacterial cell walls (i.e., β-lactams and glycopeptide antibiotics) (4).

U. urealyticum can be found in the cervix or vagina (or both) of 40%-80% of the sexually mature asymptomatic women (5), but has also been linked to nongonococcal urethritis (NGU), arthritis, meningitis, chorioamnionitis, and preterm labor (6, 7). Patients suffering from clinical chorioamnionitis may deliver prematurely. *U. urealyticum* is often isolated from preterm infants, stillborn, and spontaneously aborted fetuses (5).

Most laboratories lack the capability to cultivate and test for sensitivity *Ureaplasma* spp. *and Mycoplasma* spp., as they are fastidious cell wall-free bacteria. Even if cultured, a distinction between *U. urealyticum* and *Ureaplasma parvum* is difficult to be made and results are often mistakenly reported (8).

Polymerase chain reaction (PCR) is a molecular biology method presenting high sensitivity and rapidity compared with culture-based and other traditional methods employed in STI diagnosis. However, inappropriate reporting in regard to species differentiation can occur in qualitative PCR assays as well (8, 9).

The three classes of antibiotics active against *Ureaplasma* spp. are the quinolones, tetracyclines and macrolides (10). Of these, tetracycline is the drug most commonly employed in the treatment of *U. urealyticum* infections (11).

The first tetracycline-resistant *U. urealyticum* strain was isolated in 1974 in British Columbia (12). An American study conducted in 1981 reported a 6-10% prevalence of these resistant strains (13), while another one in 1986 revealed that more than 15% of *U. urealyticum* strains isolated from STD patients were resistant to tetracycline (14). On the other hand, a frequency of only 2-3% tetracycline-resistant *U. urealyticum* strains was observed in France during the 1992-2002 interval (15). However, an increased prevalence of tetracycline and fluoroquinolone resistance in *Ureaplasma* spp. has recently been suggested in Europe (16).

In bacteria, tetracycline resistance is encoded by a number of genetic determinants. The *tetO* and *tetM* genes have been found in Gram-negative and Gram-positive bacteria, and the latter is known to be transferable between the two groups (17). *TetM* is the only tetracycline resistance determinant reported so far in mycoplasmas (18). In these bacteria, a high level of acquired resistance to tetracyclines is associated with the presence of the *tetM* gene (19) and PCR amplification identified *tetM* to be present in the tetracycline-resistant isolates (20). Our study aimed to identify *U. urealyticum* in urine samples using the PCR technique and to determine its susceptibility to tetracycline based on the presence or absence of the *tetM* gene.

Material and methods

Patients and specimens

Between January 2014 and September 2019 a number of 622 subjects aged 17 to 75 years, including patients presenting STI symptoms (genital discharge, dysuria, fever or abdominal pain) and asymptomatic individuals involved in unprotected sex with multiple partners were enrolled in this study approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca (no. 146/15.04.2014). No patient was subjected to antibiotic treatment two weeks prior to inclusion in the study.

Samples of 30 to 50 mL first void urine were collected in sterile polypropylene containers during early morning hours, provided the patients had not urinated for the preceding 4 hours.

DNA extraction

Aliquots of urine samples were centrifuged for 15 min at 15,000 g and the resulting pellet was re-suspended in phosphate-buffered saline (PBS). MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE Biotechnologies, USA) were used for DNA extraction and purification according to supplier recommendations. The DNA was suspended in nucleotide-free water.

DNA concentration and purity were determined by nano-photometer readings against a reference nucleotide-free water. DNA purification sequence was repeated whenever purity was inappropriate.

DNA amplification for U. urealyticum detection

Three µl of DNA extracts were amplified on a DNA thermal-cycler with a Seeplex® STD6 ACE Detection (Seegene, Korea) kit in a total volume including 17 µl PCR mix with 5x ST-D6ACE PM primers containing primer pairs for *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, U. urealyticum, Mycoplasma genitaliumi,* and *Mycoplasma hominis*, a primer pair for internal control and an internal control template.

PCR was initiated with a 15 min denaturation step at 94°C and a 10 min extension-step at 72°C followed the 40 amplification cycles (each including a 30 s denaturation step at 94°C, a 90 s annealing step at 63°C and a 90 s chain elongation step at 72°C). PCR products were separated using a 2% agarose gel stained with ethidium bromide.

DNA amplification for U. urealyticum confirmation and tetracycline susceptibility

DNA extracted from *U. urealyticum* positive samples was amplified using *U. urealyticum* - specific *urease* gene and *tetM* gene primers designed using a modified version of previously published sequences (10, 18, 21). The primer sequences are presented in Table 1. No reference tetracycline resistance strains were available for positive control.

Amplification was carried out in a total volume of 50 µl including 2 µl DNA extract, 25 µl My-

Table 1. The sequences of the *tetM* and *urease* genes primers

Primer	Sequence	Product length	GC%
tetM		398	
Forward	TTATCAACGGTTTATCAGG		49.74
Reverse	CGTATATATGCAAGACGTTG		52.07
urease gene		429	
Forward	ACGACGTCCATAAGCAACT		56.79
Reverse	CAATCTGCTCGTGAAGTATTAC		55.05

Taq™ Red Mix (Bioline, UK) mixed with 4 µl primers containing primer pairs for *tetM* and *urease* genes, and 19 µl water (ddH_2O).
Based on several optimization attempts, the protocol employed a 60 s denaturation step at 95°C and 35 amplification cycles including a 15 s denaturation step at 95°C, a 15 s annealing step at 58°C and a 10 s extension step at 72°C. The amplicons were separated via gel electrophoresis using 2% agarose gel (see Figure 1).

Results

U. urealyticum detection in urine samples

Two hundred of the 622 subjects included in the study were positive for various STIs, 68 of these (10.93% of the total patients) presenting the corresponding *U. urealyticum* DNA (the second most frequently detected bacterium after *C. trachomatis*), 34 of these patients presented multiple infections.

Detection of U. urealyticum tetracycline susceptibility

The *urease* gene was amplified in all the samples collected from *U. urealyticum* positive patients. The *tetM* gene was amplified in 2 samples accounting for tetracycline-resistant strains (e.g. sample S2 in Figure 1).

Discussion

In recent years, *Ureaplasma* spp. were recognized as pathogens in numerous clinical presentations. The large number of patients positive for *U. urealyticum* (34% of the positive patients) identified in our study is in accordance with literature data and the conclusions of our previous studies (7, 22, 23) indicating that infection with this bacterium remains one of the major causes of STIs in Romania.
An accurate diagnosis is needed in order to prevent severe complications and to control transmission, especially in asymptomatic infections. As bacteriological cultures allow further testing of antibiotic susceptibility, the technique is useful in detecting susceptibility to antibiotics. However, in contrast to other prokaryotes, currently there are no Clinical and Laboratory Standards Institute (CLSI) recommended guidelines for the susceptibility testing of ureaplasma. Agar disk diffusion is unsuitable for such purpose because of the small colony sizes and prolonged growth periods (24).

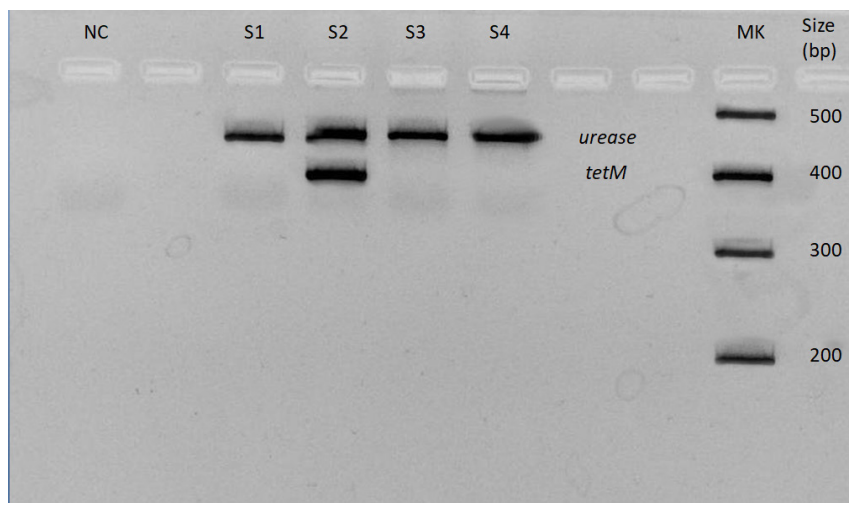

Fig. 1. Electrophoretic analysis of amplicons by PCR with specific primers for urease and tetM genes. NC: negative control, S1-S4: samples, MK: molecular weight marker, bp: base pairs

Recent years have seen an extensive usage of nucleic acid amplification tests (NAATs), for carrier detection in particular. These genetic tests enable the establishing of a diagnosis for bacterial STIs with a sensitivity of up to 99%. Other studies have also argued that the PCR technique is more sensitive, accurate and rapid compared with classical methods (9, 25, 26). NAAT testing was also proved to be efficient in typing *U. urealyticum* strains including non-cultured specimens. For detection, we employed a high specificity/ sensitivity multiplex PCR kit (Seeplex® STD6 ACE Detection) able to detect the presence of several STI pathogens in the same sample, useful since these pathogens often associate and may present similar symptoms. As a result, in our 200 positive samples we found 46 associations of two to five STI pathogens, 34 of them involving *U. urealyticum*. Faster detection of *U. urealyticum* via PCR (<24 h) compared with traditional cultures (2-5 days) is particularly important in the case of very low birth-weight infants.

Another advantage of the technique is the use of first urine jet samples as DNA source for STI detection. Urine collection is less invasive compared to the harvesting of vaginal or urethral secretions, an aspect previously reported to have influenced a significant number of subjects to avoid STI identification tests (27).

Although *Ureaplasma* have been recognized as two separate species since 2000, species discrimination is still problematic, partly due to culture-based commercial kits. In some instances *Ureaplasma* spp. are reported as *U. urealyticum* by default due to historical taxonomic reasons, negatively impacting our understanding of the influence of the two species in the clinical outcome and the distribution of resistant species (16). A recent systemic review and meta-analysis by Zhang et al. (2014) has supported the idea that *U. urealyticum* contributes to the development of NGU, whereas *U. parvum* does not (28). To confirm the presence of *U. urealyticum* in the samples initially detected as positive for this bacterium, we determined the presence of the *urease* gene in all these samples. The *urease* structural gene from *U. urealyticum* contains unique sequences that are not present in other mycoplasmas (29). The *urease* gene was amplified in all the samples collected from *U. urealyticum* positive patients, confirming the accuracy of both PCR methods employed in the study (see Figure 1).

Initially found in streptococci, the *tetM* gene often located on a conjugative transposon confers high-level resistance. Literature data suggest that *tetM* is widely disseminated in the urogenital flora. This is similar to the previously documented spread of resistance genes from the *Enterobacteriaceae* to unrelated gram-negative genera (30). The *tetM* class of determinants appears to have a wide host range, and since this determinant can be transferred by transformation or conjugation, in time, other species and genera are susceptible to acquire it (31, 32).

Tetracyclines are the first-line treatment of infections caused by *Ureaplasma* spp., to which antibiotic resistance is continuously increasing (11). Tetracycline resistance is well characterized among *Ureaplasma* spp. and is associated with the presence of the horizontally acquired *tetM* resistance gene that provides ribosomal protection (21, 33). A large percentage of microorganisms containing the *tetM* gene provide conditions for the transfer of the determinant to mycoplasma cells. Dystrophic changes in the urogenital mucosa and desquamation of the epithelium also facilitate the adhesion of microorganisms, mycoplasmas in particular (34).

A study screened the DNA isolated via PCR from 130 *Ureaplasma* spp. isolates for the presence of the *tetM* gene and identified three positive strains (2.3%). In contrast, broth culture screening for tetracycline resistance identified only two of these isolates. It therefore appears that screening for the presence of the *tetM* gene

is less likely to miss resistant isolates than broth microdilution methods employed in determining tetracycline resistance (10). A 2012 study performed on 66 strains of *Ureaplasma* spp. revealed that all tetracycline-resistant strains contained 397 bp regions of the *tetM* and the *int-Tn* genes (24). Another study concluded that *U. urealyticum* strains considered to be tetracycline sensitive were also found to lack *tetM* (18). According to Dégrange et al. (2008), phenotypically susceptible isolates bearing the *tetM* gene should be considered and reported as resistant, despite the lack of data regarding the behavior of the microorganism during *in vivo* therapy (15). As clinicians tend to focus on the practical effects of the treatment they recommend, disregarding genetic evidence of antibiotic resistance, we chose to determine the presence of the *tetM* gene in order to observe the tetracycline resistance of the identified strains.

In our study we determined a percentage of 2.94% strains resistant to tetracycline that presented the *tetM* gene. A French study conducted from 1999 to 2002 reported high (>80%) sensitivity rates to tetracycline (15), while in a 4-year-study (2012-2015) in central Greece the presence of the *tetM* gene was not detected in 76 positive cases of *Ureaplasma spp*., the provided explanation being that in this region doxycycline administration remains the first therapeutic option unless special medical circumstances dictate otherwise (35). A certain increase (7.5%) in tetracycline resistance among *Ureaplasma* spp. was observed in a 2010-2015 French study on 831 isolates (19). The number of tetracycline-resistant strains determined in our study (<10%) is thus consistent with several European studies (15, 19, 35).

This is a reassuringly low resistance level compared with other international reports, especially from outside Europe. High levels of tetracycline resistance (73%) were documented in a South African study, but speciation indicated that *U. parvum* was the predominant *Ureaplasma* spp. conferring antimicrobial resistance (36). A Cuban study reported that 31% of the analyzed *U. urealyticum* strains were resistant to tetracycline (37). Another research in Northern Greece on 100 isolates of *Ureaplasma* spp. found 14.3% *tetM*-positive isolates belonging to *U. urealyticum* (38). A very recent Tunisian study on 101 molecularly typed *Ureaplasma* spp. clinical strains detected 37.62% tetracycline-resistant isolates. In contrast to such reduced susceptibility to tetracycline, *Ureaplasma* spp. strains exhibited an extremely high sensitivity to doxycycline (39).

Study limitations include the relatively short period of time covered in this research and the low number of subjects positive for *U. urealyticum* we identified, a direct consequence of the low number of individuals tested (not a representative population). Nevertheless, this is the first such study carried out in Romania. Further research on a larger population and full access to clinical, epidemiological, and microbiological variables are needed to validate our results.

Our study proposed primer changes and a simpler method making use of devices commonly found in molecular biology laboratories. However, bacterial DNA isolated from biological samples can be subsequently used to determine susceptibility to tetracycline with the proposed technique. Certain DNA amounts can be made available for further determinations e.g. susceptibility to antibiotic resistance as the highly accurate NAATs detection method requires only small DNA amounts.

The high resistance levels reflected in international studies call for continuous surveillance in order to keep track of resistance patterns, limiting the risk of importation. Our results have demonstrated that such resistant strains can occur and need to be detected in order to provide a proper treatment. The early diagnosis and appropriate treatment of *U. urealyticum* may

prove to be important in reducing infertility or complications caused by this bacterium. Our results indicate that the non-invasive technique we employed can produce quick results both for *U. urealyticum* detection and determination of its resistance to tetracycline using a single easy-to-collect biological sample.

Abbreviations

STI: Sexually transmitted infection
CDC: Centers for Disease Control and Prevention
NGU: nongonococcal urethritis
PCR: Polymerase chain reaction
PBS: phosphate-buffered saline
CLSI: Clinical and Laboratory Standards Institute
NAAT: nucleic acid amplification test

Authors' contribution

MLV – Conceptualization, Methodology, Investigation, Validation
HVM – Validation, Data Curation, Supervision
AK – Investigation, Resources, Writing – original draft preparation
AP - Resources, Writing – review and editing, Data Curation
CAT – Resources, Writing – review and editing, Data Curation

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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