https://doi.org/10.31925/farmacia.2023.1.9 **ORIGINAL ARTICLE**

ANTIMICROBIAL RESISTANCE OF UREAPLASMA UREALYTICUM AND MYCOPLASMA HOMINIS IN THE ROMANIAN POPULATION

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Abstract

The present study aimed to assess the antimicrobial susceptibility and resistance to antibiotics available in Romania against *Ureaplasma urealyticum* and *Mycoplasma hominis* genital isolates at this time. We collected urethral swabs and vaginal samples from patients who presented at "Ponderas" Academic Hospital (Bucharest, Romania) in the Dermato-venereology Department from January 2021 until December 2021. Fifty-two samples were obtained from two groups of patients: symptomatic subjects complaining of several urogenital symptoms (i.e. burning sensation or pain during urination, vaginal/cervical discharge etc.) and completely asymptomatic subjects who came for sexually transmitted diseases (STDs) microbiological screening. We used a Mycoplasma kit to assess the presence of *U. urealyticum* and *M. hominis* and their susceptibility to antibiotics. 82% of men (n = 28) were diagnosed with *U. urealyticum* genital infection. 50% of females (n = 9) were positive for both *U. urealyticum* and *M. hominis* (p = 0.01914). All isolates (100%, n = 52) were susceptible to doxycycline and tetracycline. Most mollicutes showed significant resistance to quinolones. Mollicutes showed a better susceptibility to macrolides when only one isolate was present (76%, 73% and 84% for erythromycin, azithromycin and clarithromycin, respectively) compared to mixed isolates (7%) (p < 0.001). Multiple antibiotic resistance (MAR) index was calculated, and the result was 0.33 for *U. urealyticum* isolates, 0.64 for genital coinfection with mixed isolates, and 0.42 for *M. hominis* isolate (p = 0.0003614). In conclusion, our study analysed the antibiotic susceptibility of *U. urealyticum* and *M. hominis* in urethral and vaginal samples in the Romanian population, for a better understanding of the rational use of antibiotics, which may be helpful to avoid both treatment failure and the abuse of antimicrobial agents.

Rezumat

Acest studiu și-a propus să evalueze susceptibilitatea și rezistența la antibioticele disponibile în România la momentul actual a izolatelor genitale de *Ureaplasma urealyticum* și *Mycoplasma hominis*. S-au recoltat probe uretrale și vaginale de la pacienti care s-au prezentat la Spitalul Academic „Ponderas" (București, România) în Secția de Dermato-venerologie în perioada ianuarie - decembrie 2021. Au fost obținute 52 de probe de la două grupuri diferite de pacienți: subiecți simptomatici cu unul sau mai multe simptome uro-genitale (senzație de arsură sau durere în timpul micțiunii, secreții uretrale/vaginale/cervicale etc.) și subiecți complet asimptomatici care au venit pentru screening pentru boli cu transmitere sexuală (BTS). S-a folosit un kit special pentru a evalua prezența *U. urealyticum* și *M. hominis* și susceptibilitatea acestora la antibiotice. 82% dintre bărbați (n = 28) au fost diagnosticați cu infecție genitală cu *U. urealyticum*. 50% dintre femei (n = 9) au fost pozitive atât pentru *U. urealyticum*, cât și pentru *M. hominis* (p = 0,01914). Toate izolatele (100%, n = 52) au fost susceptibile la doxiciclină și tetraciclină. Majoritatea molicuților au arătat rezistență semnificativă la chinolone. Molicuții au arătat o susceptibilitate mai bună la macrolide atunci când a fost prezent un singur izolat (76%, 73% și, respectiv, 84% pentru eritromicină, azitromicină și claritromicină), comparativ cu izolatele mixte (7%) (valoarea p < 0,001). S-a calculat indicele de rezistență multiplă la antibiotice (MAR) și rezultatul a fost 0,33 pentru izolatele *U. urealyticum*, 0,64 pentru coinfecția genitală cu izolate mixte și 0,42 pentru izolatul *M. hominis* (p = 0,0003614). În concluzie, studiul nostru a analizat sensibilitatea la antibiotice a *U. urealyticum* și *M. hominis* în probe uretrale și vaginale din populația României, pentru o mai bună înțelegere a utilizării raționale a antibioticelor, care poate fi utilă pentru a evita atât eșecul tratamentului, cât și abuzul de agenți antimicrobieni.

Keywords: mollicutes, *Ureaplasma, Mycoplasma*, antimicrobial susceptibility, multidrug resistance

Introduction

Several *Ureaplasma* species, such as *Ureaplasma urealyticum* and *Ureaplasma parvum*, as well as *Mycoplasma hominis* belong to the mollicutes class, the *Mycoplasmatales* order [1]. Within the mollicutes class, five species represent human pathogens. *Mycoplasma pneumoniae* is the most known respiratory pathogen that is an agent of "walking pneumonia". The rest, *U. urealyticum*, *U. parvum*, *Mycoplasma genitalium* and *M. hominis* represent urogenital pathogens. To date, minimum 14 serovars have been identified: *U. parvum* comprises four serovars – UPA1, UPA3, UPA6 and UPA14, while *U. urealyticum* contains 10 serovars – UUR2, UUR4, UUR5, UUR7-13 [2]. *U. parvum* appears to be more common than

U. urealyticum as a colonizer of the female and male urogenital tracts, as well as in the neonatal respiratory tract [1, 2].

Two of the leading causes of nongonococcal urethritis in men are represented by *M. hominis* and *U. urealyticum*, having been isolated in roughly 12% of these cases. In addition, it has been reported to cause sexually transmitted reactive arthritis (Reiter's Syndrome), epididymitis, infectious kidney stones, and arthritis in hypogammaglobulinemia patients. Regarding female patients, its role in the aetiology of pregnancy complications is suggestive, inducing preterm labour, infertility, spontaneous abortion, puerperal fever and Pelvic Inflammatory Disease. Moreover, the transmission of *U. urealyticum* to the foetus or new-born may cause not only severe bronchopulmonary dysplasia but also central nervous system (CNS) infections [3].

The genome of mollicutes is minimal, presenting an intrinsic natural resistance to all β-lactam drugs (*i.e.* cephalosporins, penicillins) caused by the absence of a cell wall in their structure. Therefore, antimicrobials affecting DNA replication (quinolones) and ribosomal protein synthesis (macrolides and tetracyclines) are generally used to treat of clinically relevant mollicutes infections. Nevertheless, incomplete or erroneous treatments and overuse of drugs cause mollicutes antimicrobial to develop resistance, which is increasingly significant in several countries [1].

The objective of this study was to evaluate the antimicrobial susceptibility and resistance to antibiotics available in Romania of *U. urealyticum* and *M. hominis* genital isolates at this time.

Materials and Methods

This study was conducted from January 2021 until December 2021 at the "Ponderas" Academic Hospital (Bucharest, Romania) in the Dermato-venereology Department. Data regarding all consecutive patients providing urogenital samples for *Ureaplasma* and *Mycoplasma* detection by culture were collected and analysed. Specimens were obtained from two groups of patients: symptomatic subjects complaining of several urogenital symptoms (*i.e.* burning sensation or pain during urination, vaginal/cervical discharge etc.) and completely asymptomatic subjects who came for STDs microbiological screening (many reported sexual contact with infected individuals). Only samples collected during the first visit were considered, excluding specimens obtained during follow-up from the same patient. The sudy was approved by the Ethics Committee of "Ponderas" Academic Hospital. Written informed consent was obtained from all patients.

Urethral/vaginal swabs

Male patients were placed in a gynaecological position; they were asked to retract the penis foreskin and maintain it throughout the procedure. The MD used sterile cotton or gauze to clean the opening of the urethra at the tip of the penis. To facilitate collecting the sample, as well as to stimulate the prostatic gland secretion, prostatic massage was performed before collecting the sample. Subsequently, a cotton swab was gently inserted approximately 2 cm into the urethra and rotated. To obtain a proper sample, the test was performed at least 3 to 5 days after the last sexual intercourse and 2 hours after urinating. The swab was placed in R1 broth (Mycoplasma Gallery IST2) to initiate the isolation of mycoplasmas. For the female patients, the vaginal sample was taken by placing the patient in a gynaecological position and carefully introducing a cotton swab into the vaginal canal. No commercial lubricants or antiseptics were used. The swabs were placed in R1 broth (*Mycoplasma* Gallery IST2) to initiate the isolation of mollicutes. The liquid media for *U. urealyticum* and *M. hominis* were inoculated from the transport medium R1 (Mycoplasma IST2 kit, bioMérieux, Marcy l'Etoile, France). To perform phenotypic identification of *U. urealyticum*, urea broth was used, containing medium base (pleuropneumonia-like organism broth), urea, horse serum and yeast extract. To establish the growth of the microorganism, phenol red was added to the culture medium since it turns from red to intense raspberry red in the presence of urease and ammonium production. Likewise, a culture medium specific for *M. hominis* had arginine added. When metabolized, arginine produces an alkaline compound that turns phenol red to raspberry red. Culture media were incubated at 37°C until the phenol red indicator changed colour. The *Mycoplasma* IST2 gallery was used according to the manufacturer's instructions in the following manner. A swab with the sample was placed in the transport medium R1 broth (3 mL). The broth was mixed with the lyophilisate R2 provided by the gallery, and it contained the substrates required to develop the microorganisms. A volume of 55 mL was distributed to each of the domes of the 22 tests, divided into three sections. In the first section, phenotypic detection of *U. urealyticum* and *M. hominis* was performed. In the second section, microorganisms were quantified, determining whether the sample contained a concentration > 10^4 change in colour units (CCU)/sample. This indicated the importance of the presence of these microorganisms. In the last section, sensitivity tests were performed for different anti-microbials, and sensitivity was suggested by a change in colour from yellow to red. Lastly, after the inoculation of each of the domes, two or three drops of pure mineral oil were added. The gallery was incubated at 37°C, and the results were recorded at 24 and 48 hours [4, 5].

Statistical analysis

The categorical variables were expressed as count and percentage. Association tests were performed by chi-square or Fisher's exact test, accordingly. The statistical significance was assumed if a null hypothesis

could be rejected at a p-value of 0.05. The multiple antibiotic resistance (MAR) index was calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics to which an organism is exposed. MAR index values greater than 0.2 indicate that antimicrobial resistance is high. The MAR index values for *M. hominis* and/or *U. urealyticum* were compared to a non-parametric test: Kruskal-Wallis (p = 0.0003614), considering the normality conditions were not fulfilled (Shapiro-Wilk normality test, p = 0.0002859). The data were analysed using the R Statistical Software version 4.4.1.

Results and Discussion

The study population consisted of 52 patients (34 males and 18 females) with *U. urealyticum* and/or *M. hominis*. 28 of the male patients (82%) and 9 of the females (50%) were diagnosed with *U. urealyticum* genital infection. In contrast, 5 males (15%) and 9 females (50%) were positive for both *U. urealyticum* and *M. hominis* (Figure 1). There is a statistically significant association between gender and a particular isolate (p = 0.01914). There was only 1 male (3%) with *M. hominis*.

The average age for the *U. urealyticum* patients was 33 for male and 25 for female patients; for those who had mixed isolates, the average age was 29 for males and 32 for females. The male patient with *M. hominis* was 51 years old.

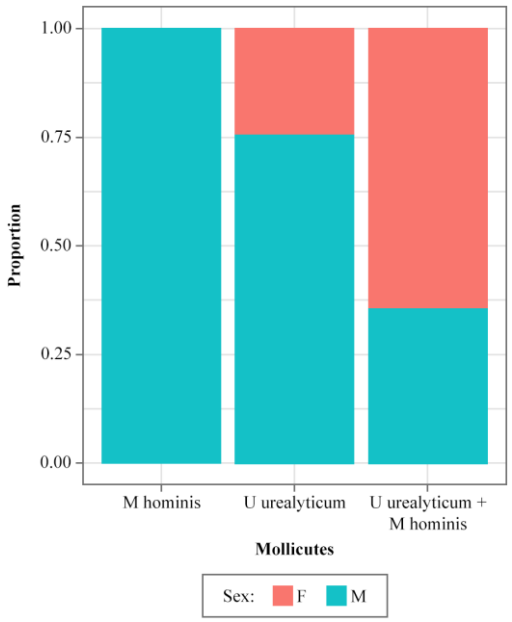

Figure 1.
Distribution of the *U. urealyticum* and *M. hominis* isolates in male and female patients

Forty-three patients (27 males and 16 females) were asymptomatic, and 9 (7 males and 2 females) experienced symptoms suggestive of balanitis, prostatitis and vaginitis, respectively. None of the women included in the study was pregnant.

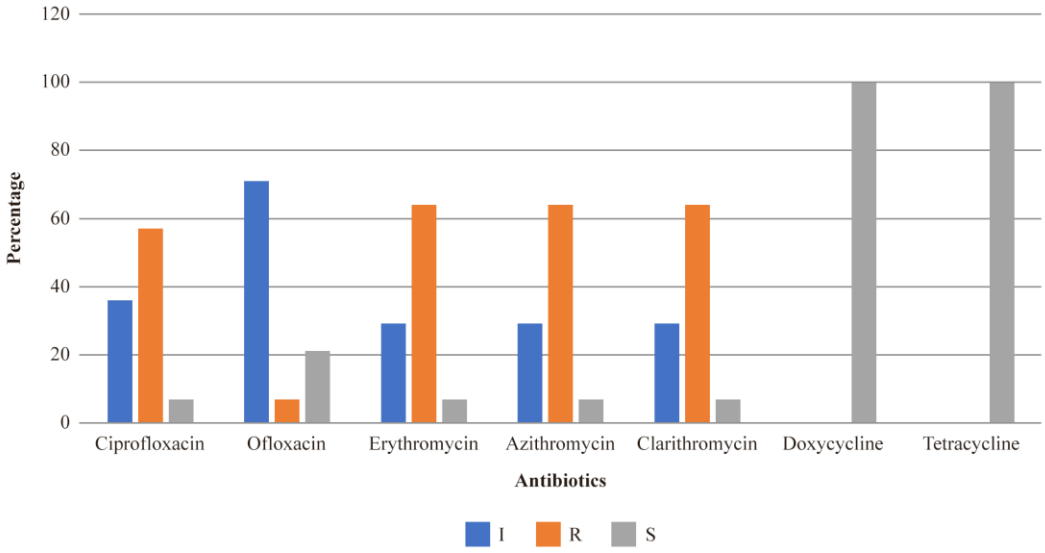

Figure 2.
Antimicrobial susceptibility of genital coinfection with both *U. urealyticum* and *M. hominis* isolates

14 out of the 52 patients with genital infections caused by *U. urealyticum* and, or *M. hominis* tested positive for coinfection with both isolates. All of them (100%) were sensitive to doxycycline and tetracycline. In addition, 21% of the patients were susceptible to ofloxacin. However, 71% of *U. urealyticum* + *M. hominis* mixed isolates showed an intermediate pattern to ofloxacin on the antibiograms, in contrast to just 7% that were resistant to ofloxacin. Mixed isolates showed extremely low susceptibility to ciprofloxacin, erythromycin, azithromycin and clarithromycin (7%). Erythromycin, azithromycin and clarithromycin presented an identical pattern on the antibiograms (susceptible in 7% of the cases, intermediate in 29%

of the patients and resistant in 64% of the cases). The second most resistant antibiotic is ciprofloxacin, with a 57% rate of resistance and a 36% rate of intermediate response. A significant association was found between the resistant strains and the type of antibiotic ($p < 0.001$). The antimicrobial susceptibility of genital coinfection with both *U. urealyticum* and *M. hominis* isolates is presented in Figure 2.

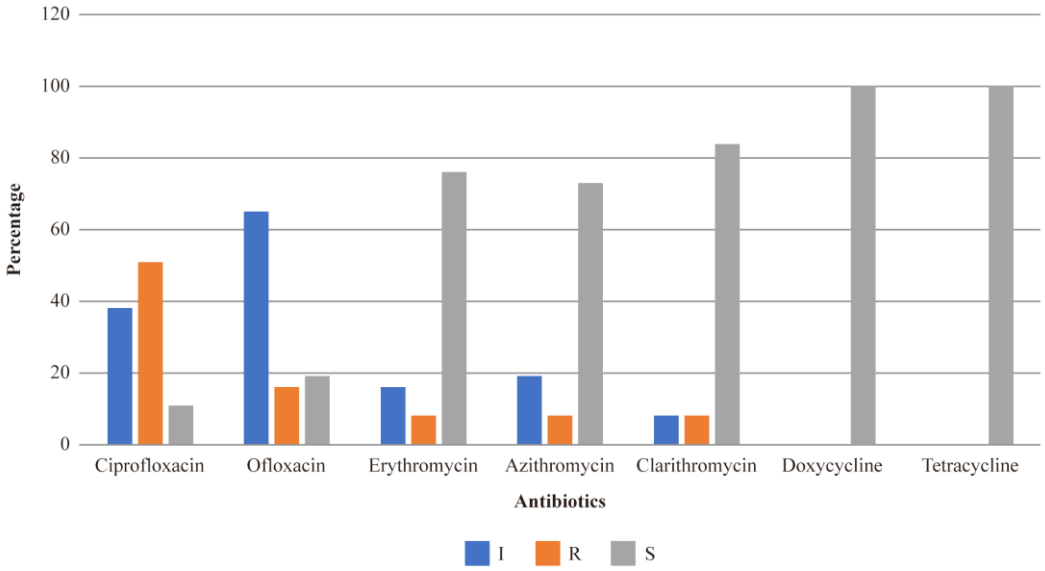

Figure 3.
Antimicrobial susceptibility of *U. urealyticum* genital isolates

Regarding the *U. urealyticum* isolates, all 37 patients (100%) were susceptible to doxycycline and tetracycline. This mollicute showed 84% susceptibility to clarithromycin, followed by erythromycin and azithromycin, with a percentage of 76% and 73%, respectively. *U. urealyticum* strains were the least susceptible to ciprofloxacin (11%) and ofloxacin (19%). Moreover, these strains were the most resistant to ciprofloxacin (51%) compared to ofloxacin (16%), erythromycin (8%), azithromycin (8%) and clarithromycin (8%). The intermediate response rates were ofloxacin (65%), ciprofloxacin (38%), azithromycin (19%), erythromycin (16%) and clarithromycin (8%). A significant association was found between the resistant strains and the type of antibiotic ($p < 0.001$). The antimicrobial susceptibility of *U. urealyticum* genital isolates is presented in Figure 3.

Out of the total of 52 patients included in the study, there was just 1 patient that tested positive for *M. hominis* genital isolate. The antibiogram exposed that the isolate was susceptible to doxycycline, tetracycline, ciprofloxacin and ofloxacin and resistant to erythromycin, azithromycin and clarithromycin.

The multiple antibiotic resistance (MAR) index has been calculated. The result was 0.33 for *U. urealyticum* isolates, 0.64 for genital coinfection with both *U. urealyticum* and *M. hominis*, and 0.42 for *M. hominis* isolates. The differences between the MAR index values in relation to the isolates are particularly statistically significant ($p = 0.0003614$) (Table I, Figure 4).

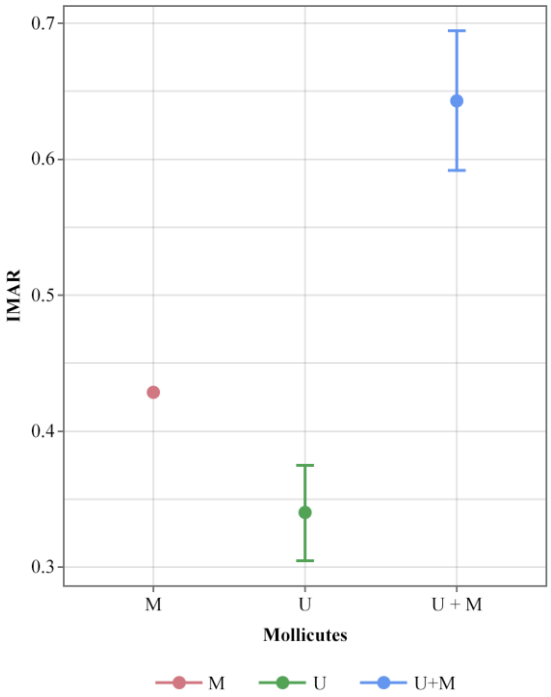

Figure 4.
Multiple antibiotic resistance index – confidence interval
(IMAR = multiple antibiotic resistance index;
M = *M. hominis* isolate; U = *U. urealyticum isolate*;
U + M = *U. urealyticum* + *M. hominis* isolates)

Table I

Numerical indicators for MAR index concerning the infection with *U. urealyticum* and, or *M. hominis*

Mollicute type	No	IMAR	sd	se	ci
M	1	0.428571	NA	NA	NaN
U	37	0.339768	0.213892	0.035164	0.071315
U+M	14	0.642857	0.192072	0.051333	0.110899

M = *Mycoplasma hominis*, U = *Ureaplasma urealyticum*, U+M = coinfection with both mollicutes, No = number of cases, IMAR = multiple antibiotic resistance index, sd = standard deviation, se = standard error, ci = confidence interval

The prevalence of *Ureaplasma* species and *M. hominis* was reported to be around 21% and 3%, respectively [6]. These are quite common genital infections in sexually active adults. Hence, knowledge of the prevalence and antimicrobial susceptibility of genital mollicutes infection in a population is fundamental in order to monitor the local drug resistance rate and to provide guidance for the rational use of antibiotics. The paper aimed to investigate the susceptibility of mollicutes to the antibiotics available in Romania at this time.

Similar to previous studies, we observed that *U. urealyticum* was the most prevalent (71%) species, isolated in samples of the urethra and the vaginal canal from the studied individuals [7]. According to the present study, men are the most affected (65%) by *U. urealyticum*, regardless of the presence of genital symptoms ($p < 0.05$). Women were more affected by genital coinfection with mixed isolates than men ($p < 0.05$), probably because of the asymptomatic vaginal colonization (Figure 1). Existing data show that women are positive more often (60 - 80%) for mollicutes than men [1, 8, 9]. These findings might be caused by the fact that men are usually less tested for infection with *U. urealyticum* and/or *M. hominis*. At the Dermato-venereology Department of the "Ponderas" Academic Hospital, we have more male patients presenting for STDs. Women tend to have their genital check-ups at the Gynaecology Department. *M. hominis* and *U. urealyticum* have been implicated in various infections that may lead to infertility. Therefore, in our opinion, every gynaecologist and dermatovenereologist should perform tests for *U. urealyticum* and *M. hominis* whenever a patient complains of genital symptoms or presents for an STD screening.

The main finding of our study was the high prevalence of mollicutes (*U. urealyticum*, *M. hominis* and *U. urealyticum* + *M. hominis*) susceptible to doxycycline and tetracycline (100%) (Table I, Figure 2, Figure 3). None of the isolates showed resistance to either doxycycline or tetracycline. As already indicated, tetracyclines and macrolides (erythromycin, azithromycin and clarithromycin) were the most active antimicrobial drugs, regardless of the mollicutes species identification. In ureaplasmas, tetracycline resistance has been reported to occur in approximately 20% of the isolates [8, 10]. However, we believe that, on the one hand, the antimicrobial susceptibility rates are different according to the geographical area and the trend in using one class of antibiotics more than others. A similar study performed in Greece reported similar results: they noticed no resistance to macrolides and tetracyclines, while most of the isolates presented resistance or moderate sensitivity to quinolones [11]. On the other hand, over the years, mollicutes have developed mutations that make them resistant to one or more antibiotics. ParC S83L was the most prevalent mutation in levofloxacin-resistant *Ureaplasma* strains, followed by ParE R448K. The two mutations, GyrA S153L and ParC S91I were commonly identified in quinolone-resistant M. hominis [12]. Mutations in 23S rRNA account for intrinsic resistance to macrolides in *M. hominis* [13]. Tetracycline-susceptible isolates, which carry the tet(M) mutation, should be reported as resistant to all tetracyclines. Screening for the tetM gene could identify tetracycline-resistant strains. However, we should pay attention to false-positive strains, which is a warning for using PCR screening for antibiotic resistance [14]. Ongoing clinical studies reported that eravacycline might be a valuable alternative to treat urogenital infections caused by human mycoplasmas and ureaplasmas, including infections caused by macrolide, tetracycline and fluoroquinolone-resistant organisms [15].

Regarding the coinfection with mixed isolates, we found that the two mollicutes have the same patterns of susceptibility and resistance to erythromycin, azithromycin and clarithromycin (S = 7%, R = 64% and I = 29%). Following the available data, quinolones (ciprofloxacin R = 57% and ofloxacin I = 71%) showed the highest rate of resistance in treating these kinds of infections [12, 16, 17]. Levofloxacin was not part of the kit used for *U. urealyticum* and *M. hominis* antibiograms available in the "Ponderas" Academic Hospital. Therefore, we agreed that the mollicutes resistant or showing an intermediate response on the antibiogram to either ciprofloxacin or ofloxacin were considered resistant to levofloxacin as well. A statistically significant association was found between the resistant strains and the type of antibiotic ($p < 0.001$). This information is essential to raise awareness against using quinolones empirically for the treatment of genital infection with *U. urealyticum* and *M. hominis* [18]. Josamycin was not included in our study since it has not been used in Romania yet. Other studies reported that, for *Ureaplasma* species, josamycin, minocycline and doxycycline were highly and equally effective (for josamycin S = 90%, I = 8% and R = 1%)

[19]. Moreover, new quinolones should also be considered (trovafloxacin, sparfloxacin, moxifloxacin, gemifloxacin and gatifloxacin) since studies have shown that mollicutes were more susceptible to them than to the classic quinolones [12, 20, 21].

Comparing the pattern of the coinfection antibiogram with that of *U. urealyticum* infection, we can infer that the percentages of the studied antibiotics leant towards a higher susceptibility of *U. urealyticum* to the same antibiotics that were used for the treatment of the coinfection with mixed isolates (clarithromycin 84%, erythromycin 76%, azithromycin 73%, ofloxacin 19% and ciprofloxacin 11%, $p < 0.001$). The high susceptibility of the *U. urealyticum* to macrolides compared to the lowest susceptibility of the *U. urealyticum* + *M. hominis* mixed isolates (7%) to the same class of antibiotics raises the question regarding the presence of some resistance factors or aggression factors inside the mollicutes' genomes; these could be identical for *U. urealyticum*, and *M. hominis* or they could be different, but they have a cumulative effect on the antibiotic susceptibility. The antibiogram of the single *M. hominis* isolates illustrated the same pattern for the other two categories.

To emphasize the differences regarding antimicrobial resistance between the coinfection with both *U. urealyticum* and *M. hominis* and the infection with *U. urealyticum*, we have also calculated the MAR index values (Table I, Figure 4). This index was 0.33 for *U. urealyticum* isolates, 0.64 for genital coinfection with both *U. urealyticum* and *M. hominis*, and 0.42 for *M. hominis* isolate ($p = 0.0003614$). MAR index values greater than 0.2 indicate that antimicrobial resistance is high. This clearly shows that patients with coinfection with mollicutes tend to be more resistant to the standard antibiotics used for mollicute infection than those with *U. urealyticum* alone. Another critical aspect to consider is the high possibility of having to treat patients who have a genital infection with mollicutes and a coexisting STD (*Enterococcus*, *Enterobacteriaceae*, *Streptococcus*, *Staphylococcus*) resistant to quinolones or tetracycline [22].

We are aware of the limitations of this study. Firstly, a small number of patients were evaluated for anti-microbial resistance. However, it should be noted that literature data showed similar results. Secondly, the "Ponderas" Academic Hospital's laboratory does not use a PCR assay to determine various *Ureaplasma* strains (*i.e. U. parvum*).

Conclusions

In conclusion, the present study retrospectively analysed the antibiotic susceptibility of *U. urealyticum* and *M. hominis* in urethral and vaginal samples in the Romanian population, aiming for a better understanding of the rational use of antibiotics, which may be helpful to avoid both treatment failure and the abuse of anti-microbial agents.

Conflict of interest

The authors declare no conflict of interest.

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