Identification and Characteristics of Multidrug-Resistant *Ureaplasma urealyticum* and *Mycoplasma hominis* Isolates among Female Patients with Recurrent Non-Gonococcal Urethritis in a Tertiary Hospital, China

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Abstract

**Background:** Concerns are increasing over the mechanisms of drug resistance pathogens among non-gonococcal urethritis (NGU) or cervicitis. The study aims to explore the distribution of *Ureaplasma urealyticum* (*U. urealyticum*) and *Mycoplasma hominis* (*M. hominis*) isolates, characteristics of multidrug resistance, and the underlying mechanism to allow clinicians to deliver rational therapy for NGU.

**Methods:** Samples from cervical secretions of 246 patients with NGU were collected. *Mycoplasma* culture and drug susceptibility tests were performed, respectively. The resistance genes were detected by multiplex Polymerase Chain Reaction (PCR), and the PCR products were cloned and sequenced. **Results:** Out of 246 samples, the overall prevalence of *U. urealyticum* and/or *M. hominis* infection was 39.02% (96/246). In 96 culture positive samples, the overall infection rate of *U. urealyticum* was higher than that of the single *M. hominis* (48.87% vs. 28.13%) (**p** < 0.05), and the coinfection rate was 25.00% (24/96). Each *U. urealyticum* and *M. hominis* isolate exhibited resistance to at least three types of antimicrobial agents (manifest by more than 70% resistance to erythromycin, tetracycline, ciprofloxacin, and spiramycin, followed by ofloxacin, roxithromycin, azithromycin and josamycin (with resistance thereto of more than 50%). More than 80% of *U. urealyticum* and *M. hominis* isolates were susceptible to minomycin and doxycycline, whereas 81.16% and 77.44% of both *U. urealyticum* and *M. hominis* isolates were also susceptible to minomycin and doxycycline. Surprisingly, the resistance rate of the mixed infection was higher than that of erythromycin, tetracycline and ciprofloxacin comparison to the single infection (**p** < 0.05).

All tetracycline-resistant isolates carried the tetM gene and 50% of erythromycin-resistant isolates carried the ermA gene. **Conclusions:** Among outpatients with recurrent NGU, the *U. urealyticum* infection dominated, followed by *M. hominis*, mixed *U. urealyticum* and *M. hominis* infection. Minomycin and doxycycline are recommended for empirical clinical treatment. The determination of *U. urealyticum* and *M. hominis* infection, antibiotic susceptibility testing is crucial for effective therapy.

**Keywords:** non-gonococcal urethritis (cervix); mycoplasma; infection; drug resistance

1. Introduction

Non-gonococcal urethritis (NGU) or cervicitis is one of the most common sexually transmitted diseases [1]. The main pathogens are *Ureaplasma urealyticum* (*U. urealyticum*) and *Mycoplasma hominis* (*M. hominis*) in addition to *Chlamydia trachomatis* [2]. If the treatment is not timeous, NGU is apt to induce chronic prostatitis, or epididymitis, in men. In addition, both *U. urealyticum* and *M. hominis* were associated with endometritis, salpingitis, or pelvic inflammatory disease in women. Meanwhile, they have been suggested to be related to infertility [3]. Tetracycline, quinolones, and macrolides are the first-choice drugs used to treat *Mycoplasma* spp. and *Ureaplasma* spp. infection [4]. In recent years, *Mycoplasma* spp. and *Ureaplasma species* resistant to these drugs are increasing due to the abuse of antibiotics or irregular treatment, mixed infection, or repeated infection by pathogens and other factors, especially with regard to increasing multidrug-resistant isolates, resulting in NGU infection recurrent or persist [5,6]. Persistent or recurrent NGU due to multidrug-resistant *Mycoplasma* and *Ureaplasma* infection has become a difficult problem facing those in clinical practice [7,8]. Therefore, the study aimed to explore the drug-resistance mechanism of *U. urealyticum* and *M. hominis* isolates in China; the selection of sensitive drugs is the key to effective control of *Mycoplasma* infection.

In this study, *U. urealyticum* and *M. hominis* isolates from 246 cases of recurrent NGU with resistance to 12 antimicrobial agents and the mechanism of drug resistance were analyzed. The results offer a reference for clinicians to make the right choice of antimicrobial agents as best possible.
2. Clinical Data and Methods

2.1 Patients

We conducted a retrospective study of U. urealyticum and M. hominis-infected NGU patients at an affiliated hospital of Beihua University, China. 246 patients were enrolled in a gynecology outpatient clinic and sexually transmitted disease clinics during the period from June 2020 to June 2022. Clinical and microbiological data were recorded from the database. All of them had a history of sexual confusion or urethritis symptoms. Single U. urealyticum and M. hominis (or both) were positive after mycoplasma culture at the initial diagnosis. However, gonococcus, chlamydia, Candida, trichomonad, and other urogenital tract infections were excluded through clinical and laboratory tests. Empirical single antibiotic, or a combination of two antibiotics, for prophylaxis of NGU were administered with one month, and presence of co-infection with Chlamydia trachomatis was also excluded. Those with U. urealyticum and M. hominis combined with culture could still be positive with history of previous antibiotic prophylaxis for longer than two months could be included in the study. No repetitive isolates from a single patient were included. This study, entailing collection of clinical samples, was approved by the institutional ethics committees of the participating hospital and prior written informed consent was gained from all participants in the study.

2.2 Sample Collection

Firstly, a cotton swab was used to wipe away the excessive mucus in the cervix after the patient exposed the cervix, discarded it, and then another sterile swab was inserted into the cervix to a depth of 1 to 2 cm. After remaining in place for 10 to 30 seconds, the swab was slowly rotated it through one complete turn. The sample thus collected was rapidly inoculated into liquid medium and sent to the laboratory as soon as possible.

2.3 Cultivation and Detection of Resistance to 12 Antibiotics Agents

A mycoplasma identification verification and antibiotic susceptibility testing kit (RenFuBoSai, Co., Ltd, Zhengzhou, Henan, China) was used for the culture, identification, and antibiotic susceptibility assay according to the instructions supplied. The collected cotton swabs were washed in the culture medium, and 0.1 mL of culture medium was directly inoculated into 24 wells (50 µL per well), and two drops of sterile mineral oil were overlaid thereon. After inoculation, the strips were incubated at 37 °C in an incubator. The culture time for U. urealyticum was approximately 24 to 48 h, and that for the M. hominis 48 to 72 h whereupon any changes in color could be observed. Positive results were judged to be U. urealyticum positive and M. hominis positive at different times while the color of the culture medium changed from yellow to pink.

Regarding drug susceptibility, a panel of 12 antimicrobial agents including doxycycline, minomycine, tetracycline, ciprofloxacin, levofloxicacin, ofloxacin sarfloxacin, erythromycin, spiramycin, josamycine, roxithromycin, and azithromycin could be determined by two concentration wells of each agent. Interpretation of drug susceptibility: both wells were sensitive if yellow, low-concentration if one well was red, high-concentration if one well was yellow for the intermediary, and both wells were red, indicative of drug resistance.

2.4 Detection of Related Resistance Genes by Multiple Polymerase Chain Reaction (PCR)

500 µL of broth were collected into Eppendorf tubes; these were centrifuged at 12,000 rpm for 2 minutes, we discarded the supernatant, added 500 µL double-distilled water, placed in a metal water bath at 98 °C for 10 minutes, and immediately placed the samples in a refrigerator at –20 °C for 10 minutes. Samples were then centrifuged at 12,000 rpm for 5 min. The supernatant was used as a DNA template.

A series of primers for identification of resistance genes including erm and tet were designed (Table 1). A 50-µL reaction mixture of multiple PCR amplification included 5 µL of PCR master-mix and 1.5 µL of each primer (2.5 mM). 1.5 µL (25 µg/µL) of DNA extracts were used as templates and double-distilled water was supplemented to 50 µL. Two standard strains of ATCC 33175 and ATCC 15488 were used as positive controls, and ATCC 25922 was used as a negative control. Reactions were performed in 96 optical well plates and run in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA).

PCR steps were run at 94 °C for pre-denaturation for 5 min, followed by 30 cycles of denaturation for 30 s at 94 °C; the annealing temperature and conditions (Table 1) were applied for 30 s, followed by extension for 1 min at 72 °C and final extension at 72 °C for 5 min. PCR amplicons were done under electrophoresis on 2% (w/v) agarose gel in TBE buffer at 70 V for 60 min. Gels were stained and the band size was estimated using a DNA Marker with a molecular weight of 100 to 1200 bp as a reference.

2.5 Cloning and Sequencing of PCR Amplicons

The target fragments of the PCR products were excised using a sterile scalpel. A gel kit for extraction of DNA (Promega, Madison, WI, USA) was utilized to purify the PCR products extracted from the gel. Then the PCR products were ligated into the pGEM-T® Easy vector (Takara, Shiga, Japan) and transformed into E. coli DH5α competent cells. Nucleotide sequencing was conducted directly on the cloned fragments using services provided by Sangon Biotech Co., Ltd (Shanghai, China). The sequencing results were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and compared to the GENBANK database.
Table 1. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
<th>Expected length</th>
<th>PCR condition</th>
</tr>
</thead>
</table>
| 1   | ermA    | F-AAGCGGTAAACCCCTCTGA
R-TTCGCAAATCCCCCTCTCAAC | 190             | 56 60          |
| 2   | ermC    | F-AAATCGTCATTCTGCACTG
R-TAATCTGGAAATACGGGTTTG | 299             | 56 60          |
| 3   | tetA    | F-GCTACACCTGCTTGCCT
R-CATAGATCGGCGTGTAAGGA | 410             | 56 60          |
| 4   | tetB    | F-TTGGGTAGGGGCAAAGTTTG
R-GTAATGGGGAATAAACC | 320             | 56 60          |
| 5   | tetM    | F-AGTGGAGCGATTACAGAA
R-CATATGTCCTGCGTGTC | 260             | 56 60          |
| 6   | tetK    | F-GTAAGCACAATAGGTAATAG
R-GTAATGCAATAAACCTGCTTA | 159             | 56 60          |

2.6 Statistical Analysis

SPSS 10.0 statistical software (IBM Corp., Chicago, IL, USA) was used to compare the differences in *U. urealyticum* and *M. hominis* infection and drug resistance rates using the $\chi^2$ test.

3. Results

3.1 Epidemiological Characteristics of Patents and Isolates

All of patients had a course of disease of 3 to 26 months. They had an average age of 28.14 ± 3.5 years, a minimum age of 19 years and a maximum age of 59 years. Out of 246 samples, the overall prevalence of *U. urealyticum* and/or *M. hominis* infection was 39.02% (96/246). Of the 96 samples with a positive culture, the infection rate of *U. urealyticum* was higher than that of single *M. hominis* (46.87% vs. 28.13%) ($p < 0.05$), and the coinfection rate was 25.00% (24/96).

3.2 Antimicrobial Susceptibility Profiles

This research compared the antimicrobial susceptibility patterns of the individual *U. urealyticum*, *M. hominis* and both *U. urealyticum* and *M. hominis* isolated from 96 culture positive samples. Our findings showed that all *U. urealyticum* and *M. hominis* strains exhibited resistance to at least three kinds of antimicrobial agents (manifest as more than 70% resistance to erythromycin, tetracycline, ciprofloxacin, and spiramycin, followed by ofloxacin, roxithromycin, azithromycin, and josamycine (with more than 50% resistance thereto)). More than 80% of *U. urealyticum* and *M. hominis* strains were susceptible to minomycin and doxycycline, whereas 81.16% and 77.44% of both *U. urealyticum* and *M. hominis* strains were susceptible to minomycin and doxycycline. Surprisingly, the resistance rate under mixed infection was higher than that of erythromycin, tetracycline, and ciprofloxacin compared to that under single infection ($p < 0.05$, Table 2).

3.3 Detection and Sequencing of Drug Resistance Genes

The total DNA of multidrug-resistant *U. urealyticum* and *M. hominis* as a template, the tetracycline and erythromycin resistance genes could be detected by multiplex PCR. PCR products were sequenced and BLAST analysis from GenBank showed that the DNA sequences from *tetM* and *ermA* gene were 100% identical to those described in GenBank (data not shown). Both *tetM* and *ermA* genes were successfully amplified in all tetracycline-resistant, and 50% of erythromycin-resistant *U. urealyticum* and *M. hominis* strains (Fig. 1).

4. Discussion

The aetiology of recurrent or persistent NGU shows a multi-factor nature. Infectious agents including *U. urealyticum* and *M. hominis* isolates accounted for less than 30% worldwide [9]. Ideally, treatment with etiological diagnosis should be effective and incur only low-level side-effects. *Mycoplasmas* are intrinsically resistant to β-latam antibiotics in addition to glycopeptides because of the ab-
The increasing number of drug-resistant microorganisms is a serious concern due to their widespread use as first-line treatment. The reason for the absence of cell walls and susceptibility to antibiotics lies in the irregular use of antibiotic-resistant strains, especially in sexually active periods.

In the present study, the overall prevalence of *U. urealyticum* and/or *M. hominis* infection was 39.02% (96/246) out of 246 samples and multidrug-resistant *U. urealyticum* and *M. hominis* infection led to NGU recurrence or persistence among NGU patients. This finding is consistent with results reported by other studies [12,13]. In fact, women are prone to infection with *U. urealyticum* and *M. hominis* strains, especially in sexually active periods. *Mycoplasma* spp. and *Ureaplasma* species were found in the female urogenital system as a part of the normal flora. When the body was in a normal condition, there are no clinical symptoms. When the patient’s immunity was low and the patient’s genitourinary environment changed, the number of mycoplasmas would increase correspondingly and achieve the effect of pathogenicity, and more reports indicate that mycoplasmas are prone to infection with *M. hominis*, and their findings showed that the rate of *M. hominis* infection was 48.2% in women. Similar to *M. hominis*, *Mycoplasma genitalium* (*M. genitalium*) is also part of the normal flora, however, it is difficult to culture the organism. Besides, evidence for any treatment of persistent or recurrent NGU should cover *Mycoplasma, Chlamydia trachomatis*, and even flora associated with bacterial vaginosis. Sarier et al. [17] reported that the quantification of the *Mycoplasma* load may provide a useful information for differentiating NGU infection or compensation. Generally, higher organism load (>1000 copies/mL of FVU) of *U. urealyticum* and *M. hominis* may be a predictor of NGU. More analytical assays are required for *Mycoplasma* spp. and *Ureaplasma* spp.

Tetracycline was the first-choice for treatment with *U. urealyticum* and *M. hominis* infection as well as erythromycin [18,19]. In recent years, reports of resistance to tetracyclines and erythromycin have increased year-on-year. The average *U. urealyticum* resistance rate to tetracycline increased from 46.4% in 2000 to 68.3% in 2020 [18,20]. The resistance rate of doxycycline is generally maintained at 35%, and it can, at time of writing, be used as a tetracycline-replacement drug. The resistance to tetracycline is mainly mediated by the membrane-associated tet protein, which acts mainly by exchanging protons with the cation complex of tetracycline [21,22]. All tet proteins belong to the MF protein family, and at least 18 tetracycline active efflux system proteins have been recorded in bacteria [23]. The four efflux system protein genes including tetA, tetB, tetK, and tetM were detected in the study. The tetracycline-resistant strains were all positive for tetM, which was consistent with the drug-resistant phenotype. It was caused by the expression of ribosome-protected tetM protein. tetM is the most popular active efflux system in *U. urealyticum* isolates, as reported elsewhere [24,25]. Additionally, the erythromycin-inactivating enzyme ermA gene, which is highly tolerant to erythromycin, was detected in erythromycin-resistant *U. urealyticum* and *M. hominis* isolates. The ermA gene expresses erythromycin ribosome methylation whose enzyme methylates erythromycin ribosomal target sites. The literature reports that bacteria resistance to erythromycin was related to the chemical structure of macrolides [26]. Interestingly, 50% of erythromycin-resistant *U. urealyticum* and *M. hominis* isolates carried the ermA gene.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th><em>U. urealyticum (n = 45)</em></th>
<th><em>U. urealyticum + M. hominis (n = 24)</em></th>
<th><em>M. hominis (n = 27)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (%)</td>
<td>I (%)</td>
<td>R (%)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>87.01</td>
<td>0.75</td>
<td>12.24</td>
</tr>
<tr>
<td>Minomycin</td>
<td>84.72</td>
<td>2.80</td>
<td>12.48</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2.38</td>
<td>20.23</td>
<td>77.39</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>15.08</td>
<td>11.04</td>
<td>73.88</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>22.41</td>
<td>18.42</td>
<td>59.17</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>20.54</td>
<td>19.28</td>
<td>60.18</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>21.87</td>
<td>16.78</td>
<td>61.35</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>20.45</td>
<td>6.31</td>
<td>73.24</td>
</tr>
<tr>
<td>Josamycine</td>
<td>23.53</td>
<td>0.13</td>
<td>67.25</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>25.37</td>
<td>26.54</td>
<td>48.18</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>15.04</td>
<td>12.67</td>
<td>72.29</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>20.63</td>
<td>30.13</td>
<td>49.24</td>
</tr>
</tbody>
</table>

S, Sensitivity; I, Intermediate; R, Resistance.

Table 2. Drug susceptibility of *U. urealyticum* and *M. hominis* isolated from female patients with recurrent nongonococcal urethritis.
In the present study, all *U. urealyticum* and *M. hominis* isolates were recognized as exhibiting multidrug-resistance. They exhibited resistance to at least three kinds of antimicrobial agents (manifest as more than 70% resistance to erythromycin, tetracycline, ciprofloxacin, and spiramycin, followed by ofloxacin, roxithromycin, azithromycin, and josamycine (with resistance thereto of more than 50%). More than 80% of *U. urealyticum* and *M. hominis* strains were susceptible to minomycine and doxycycline, whereas 81.16% and 77.44% of both *U. urealyticum* and *M. hominis* strains were susceptible to minomycine and doxycycline. Surprisingly, the resistance rate of the mixed infection was higher that of erythromycin, tetracycline, and ciprofloxacin when compared to commensurate rate under single infection. Besides, *U. urealyticum* and *M. hominis* isolates were highly resistant to ciprofloxacin. Fluoroquinolone antibacterial agents are among the most rapidly developed, chemically synthesized antibacterial agents in the past 20 years, and they are widely used in animal husbandry and medical applications [27,28]. Studies have found that mutations in the DNA gyrase gene located on the *Mycoplasma* chromosome cause the DNA gyrase gene concerning antimicrobial agents to change, thereby reducing drug accumulation and drug resistance, which is the cause of quinolone resistance [29,30].

Our findings reported the multidrug *U. urealyticum* and *M. hominis* isolates among female NGU patients in Eastern and Northern China and reflected certain regional profiles therein. On the one hand, female mucopurulent non-gonococcal cervicitis and urethritis are equivalent, on the other hand, the incidence of female infertility in China is increasing year-on-year. Infertility is a serious threat to women’s health. The spectrum of drug-resistance in *Mycoplasma* is constantly changing. *U. urealyticum* and *M. hominis* culture, plus drug susceptibility testing, can offer good guiding significance for clinical treatment [31,32]. However, there were some limitations to the present research: first, the sample size was small, with some selection bias therein; second, we could not conduct laboratory screening to detect *M. genitalium* and *Chlamydia trachomatis*. Besides that, we could not quantitatively evaluate *Mycoplasmas* in the genital tract. Next, more data from multiple centers should be included and in-depth exploration of treatment could be conducted in future research.

5. Conclusions

Among outpatients with recurring NGU, the *U. urealyticum* infection dominated among *Mycoplasma* infections, followed by *M. hominis*, and mixed *U. urealyticum* and *M. hominis* infection. Minomycine and doxycycline are recommended for empirical clinical treatment. In the determination of *U. urealyticum* and *M. hominis* infection, antibiotic susceptibility testing is crucial for effective therapy.

**Availability of Data and Materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Author Contributions**

LM conceived, designed the experiments and wrote a draft manuscript. BL and ML analyzed, interpreted the results of the experiments and revised the manuscript. WC performed the experiments. PL and XF collected the clinical data. All authors read and approved the final manuscript.

**Ethics Approval and Consent to Participate**

Ethical approval for collecting clinical samples was received by the institutional ethics committee of the participating hospital (Ethical approval number: Protocol Number 2020-02-05). Informed consent forms were reviewed and signed by all participants before samples collection.

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**


