Antibacterial Resistance in *Ureaplasma* Species and *Mycoplasma hominis* Isolates from Urine Cultures in College-Aged Females

Marissa A. Valentine-King,a Mary B. Browna,b

Department of Environmental and Global Health, College of Public Health and Health Professions, University of Florida, Gainesville, Florida, USAa; Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, Florida, USAb

ABSTRACT  Urinary tract infections (UTIs) affect nearly 20% of women age 15 to 29 and account for an estimated $3.5 billion in costs. Antibiotic resistance prolongs UTI treatment, and resistance profiles vary regionally. This regional variation is an important consideration in guiding empirical treatment selection. Regional studies in the United States have identified tetracycline resistance in over one-third of *Ureaplasma* species isolates, but no studies have evaluated antibiotic resistance levels in college-aged women with a first-time UTI. We tested a panel of antibiotics and determined the MICs of *Ureaplasma* species (60 *U. parvum* and 13 *U. urealyticum*) and 10 *Mycoplasma hominis* isolates obtained from urine from college-aged women with a first-time UTI. Low antibiotic resistance was found in this population of women with a first-time UTI. All *M. hominis* and *U. urealyticum* isolates were sensitive. However, two *U. parvum* isolates were resistant, with one to levofloxacin (MIC, 4 μg/ml) and one to tetracycline (MIC, 8 μg/ml). For the *Ureaplasma* spp., the MIC90s were highest against gentamicin (21 μg/ml) and lowest against doxycycline (0.25 μg/ml). In a comparison of MIC levels between *Ureaplasma* spp., *U. urealyticum* had significantly higher MICs against each antibiotic except doxycycline. For the resistant isolates, the genetic mechanisms of resistance were determined. PCR amplification identified tetM to be present in the tetracycline-resistant isolate and an S83W mutation within the parC gene of the quinolone-resistant isolate. To our knowledge, this study is the first to provide molecular and phenotypic evidence of the S83W *parC* mutation conferring levofloxacin resistance in *U. parvum* isolated from a patient in the United States.

KEYWORDS  *Ureaplasma*, *Mycoplasma*, UTI, antibiotic resistance, urinary tract infection

Urinary tract infections (UTIs) serve as a common source of morbidity: in 2007, UTIs accounted for 10.5 million U.S. ambulatory health care visits and nearly one-fifth of all emergency room visits (1). UTIs disproportionally impact women over men: data indicate that the lifetime risk of UTI exceeds 50% in U.S. women but reaches only 13% in men (2, 3). Among women, those between the age range of 15 and 29 experience the highest frequency of cases, nearing 20% (4). Women with UTI report severe discomfort or pain due to lower UTIs (5). Another study echoed these findings and found that women with UTIs reported significant impacts in all areas of quality of life, particularly in the following categories: role limitation due to physical health, social functioning, and physical pain (6).

The emergence of antibiotic-resistant UTIs complicates treatment, as it prolongs illness, limits therapeutic options, and adds to health care costs (7). As physicians in
outpatient settings typically treat UTIs empirically and antibiotic resistance patterns vary regionally, the Infectious Disease Society of America recommends that physicians rely on local antibiotic resistance data when prescribing antibiotics (8). Even if physicians send specimens for culture and sensitivity testing, most laboratories lack the capability to cultivate Ureaplasma spp. and Mycoplasma hominis, which are fastidious bacteria that lack a cell wall. Lacking this structural component causes Ureaplasma spp. and M. hominis to have intrinsic resistance to beta-lactam antibiotics and vancomycin. Further narrowing treatment options, Ureaplasma spp. and M. hominis also possess intrinsic resistance to typical UTI treatment options, including sulfonamides and trimethoprim, as well as rifampin (9). Thus, antibiotic resistance in mycoplasmas poses even further treatment challenges due to the already limited spectrum of antibiotics capable of treating them.

Ureaplasma spp. are known for their etiological role in male urethritis, urinary calculi, and adverse pregnancy outcomes and neonatal complications (9–13). Although implicated in many pathological outcomes in pregnant women and neonates, Ureaplasma spp. also colonize many healthy women, and their role in UTIs is poorly understood. Unfortunately, many of the studies investigating the role of Ureaplasma spp. and UTIs in women only assessed antibiotic treatment and failed to include controls (14–16).

Few studies have evaluated antibiotic resistance in Ureaplasma spp. in the United States; however, among those which have, concerning levels of tetracycline resistance (TET+) were detected. Two distinct studies evaluating antibiotic resistance in clinical isolates of Ureaplasma spp. obtained from either adult urogenital tract or pediatric respiratory isolates identified TET+ in 33% and 34% of samples (17, 18). A separate study identified tetM, a gene associated with TET+ and TET+ induction, in 45% of samples (9). In contrast, a study conducted in Minnesota found minimal levels of levofloxacin (4%) and tetracycline (0.4%) resistance and no erythromycin resistance among 250 clinical isolates (19). However, these previous studies evaluated antibiotic resistance in isolates collected from adults with urogenital tract infections or from neonates with respiratory infections. No previous studies have evaluated antibiotic resistance in urinary isolates alone or within college-aged women, a population at high risk for UTIs.

In this study, we constructed an antibiogram from urinary isolates collected from college-aged women presenting with a first-time UTI in Florida (20). Because this population was less likely to have chronic diseases, we predicted that antibiotic resistance levels would be less than 10% to any antibiotic. Because previous studies in the southeastern United States have identified elevated levels of TET+, we expected higher resistance to this antibiotic. When resistance was present, we identified the molecular mechanisms associated with those isolates, and we identified a quinolone-resistant Ureaplasma sp. with an S83W mutation in parC that previously has not been reported in the United States.

RESULTS

The prospective cohort study (20) recruited 180 women with symptomatic first-time UTIs that met inclusion criteria, along with 80 age-matched controls. Within the UTI group, 35/180 (19%) women had recurrent UTI episodes(s), with 9/35 (26%) experiencing two or more recurrent UTIs within a year of the initial UTI. Although Escherichia coli was the most common uropathogen (20), 53% (n = 95) of women with a first-time UTI and 51.4% (n = 18) of recurrent UTI cases had Ureaplasma sp. or M. hominis isolated as a primary or secondary uropathogen. Overall, 10 M. hominis and 73 Ureaplasma spp. were available for antibiotic sensitivity testing after recultivation from archived cultures. Of the 73 UTI episodes, 12 (16%) had no pathogen other than Ureaplasma spp. isolated; therefore in this group, Ureaplasma spp. are the most likely cause of the symptoms observed. An additional 26 episodes (36%) had high levels of Ureaplasma spp., as well as another recognized uropathogen, most commonly E. coli. In this group, Ureaplasma spp. were most likely a contributing cause rather than the primary agent. Finally, in 35 (48%) episodes, other uropathogens were predominant, and Ureaplasma spp. were present in low numbers and unlikely to be the primary cause of symptoms. Because
isolates but it does not establish a true breakpoint for isolate MICs fell below the published positive bacteria (from reductionist evolution and form a distinct phylogenetic lineage within Gram-

**TABLE 1** Summary of MIC results for *Ureaplasma parvum* and *U. urealyticum* clinical isolates

<table>
<thead>
<tr>
<th>Drug used by species</th>
<th>MIC data (µg/ml)</th>
<th>No. (% of resistant isolates)a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>U. parvum</strong> (n = 60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azithromycinb</td>
<td>0.5–2</td>
<td>1</td>
</tr>
<tr>
<td>Chloramphenicolb</td>
<td>0.5–2</td>
<td>1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.5–16</td>
<td>2</td>
</tr>
<tr>
<td>Clindamycinc</td>
<td>0.22–3.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5–4</td>
<td>1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.25–2</td>
<td>0.25</td>
</tr>
<tr>
<td>Gentamicinc</td>
<td>1–21</td>
<td>10</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.25–4</td>
<td>0.5</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25–8</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**U. urealyticum** (n = 13)

<table>
<thead>
<tr>
<th>Drug used by species</th>
<th>MIC data (µg/ml)</th>
<th>No. (% of resistant isolates)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycinb</td>
<td>0.5–4</td>
<td>2</td>
</tr>
<tr>
<td>Chloramphenicolb</td>
<td>1–4</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2–4</td>
<td>4</td>
</tr>
<tr>
<td>Clindamycinc</td>
<td>1.8–28.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1–8</td>
<td>4</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Gentamicinc</td>
<td>10–42</td>
<td>21</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.5–1</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.25–1</td>
<td>1</td>
</tr>
</tbody>
</table>

aResistance determined by using CLSI interpretive guidelines. NA, not applicable, as there are no established breakpoints. Breakpoints are available only for levofloxacin at ≥4 µg/ml, erythromycin at ≥16 µg/ml, and tetracycline at ≥2 µg/ml.

bCompounds tested at doubling dilutions from 64 to 0.5 µg/ml; all others were tested from 64 to 0.25 µg/ml.

cEnd MICs adjusted to account for drug potency.

many of the antibiotics routinely used to treat UTIs in women are ineffective against *Ureaplasma*, these microbes could contribute to treatment failure even if they were not the primary cause of symptomology. Further differentiation of *Ureaplasma* species isolates by PCR identified 60 *U. parvum* isolates, 12 *U. urealyticum* isolates, and one isolate that was a mixture of *U. parvum* and *U. urealyticum*. This mixed isolate was sensitive to all antibiotics tested, so subcloning was not performed.

The Clinical and Laboratory Standards Institute (CLSI) has established breakpoints for resistance only for levofloxacin (*Ureaplasma*, ≥4 µg/ml; *M. hominis*, ≥2 µg/ml), clindamycin (*M. hominis*, ≥0.5 µg/ml), erythromycin (*Ureaplasma*, ≥16 µg/ml), and tetracycline (*Ureaplasma*, ≥2 µg/ml; *M. hominis*, ≥2 µg/ml). Therefore, while MICs could be determined for the other antibiotics tested, technically, resistance could not be reported. Less than 3% of *Ureaplasma* isolates had antibiotic resistance to the tested antimicrobial agents with established CLSI breakpoints. Only two clinical isolates of *Ureaplasma* spp. were resistant, with one to levofloxacin (MIC, 4 µg/ml; ciprofloxacin MIC, 16 µg/ml) and one to tetracycline (MIC, 8 µg/ml; doxycycline MIC, 2 µg/ml) (Table 1). Both resistant isolates were classified as *U. parvum* by PCR. No *Ureaplasma* species isolates demonstrated resistance against erythromycin.

For the antibiotics that did not have established breakpoints, we compared our data against CLSI-established breakpoints for *Staphylococcus aureus*. *Ureaplasma* spp. arose from reductionist evolution and form a distinct phylogenetic lineage within Gram-positive bacteria (21). *S. aureus* has established CLSI breakpoints for the six antibiotics not validated in *Ureaplasma*. Additionally, the CLSI breakpoints for *Enterococcus* spp. (22) were the same as those for *S. aureus*, suggesting this was a reasonable guideline, but it does not establish a true breakpoint for *Ureaplasma*. All *Ureaplasma* species isolate MICs fell below the published *S. aureus* resistance breakpoints for azithromycin (MIC, ≥8 µg/ml) and chloramphenicol (MIC, ≥32 µg/ml) (22). However, MICs at or above the *S. aureus* resistance breakpoints were observed for clindamycin (5 *U. urealyticum* isolates, ≥4 µg/ml), ciprofloxacin (4 *U. parvum* and 9 *U. urealyticum* isolates, ≥4 µg/ml), and gentamicin (1 *U. parvum* and 9 *U. urealyticum* isolates, ≥16 µg/ml) (22). The
majority of isolates that exceeded the S. aureus breakpoints for ciprofloxacin (69%), clindamycin (100%), and gentamicin (90%) were U. urealyticum.

In a comparison of MIC data between species, U. urealyticum isolates had significantly higher MICs than U. parvum for all antibiotics except doxycycline (Fig. 1). However, regardless of species, isolates were most sensitive to doxycycline (MIC$_{90}$ ≤0.25 μg/ml), followed by tetracycline (MIC$_{90}$, U. parvum, 0.5 μg/ml; U. urealyticum, 1 μg/ml) and levofloxacin (MIC$_{90}$, 1 μg/ml), and least sensitive to gentamicin (MIC$_{90}$, U. parvum, 10 μg/ml; U. urealyticum, 42 μg/ml) (Table 1).

Among the M. hominis isolates, no antibiotic resistance was observed for the three antibiotics with established breakpoints (clindamycin, levofloxacin, and tetracycline). M. hominis isolates (Table 2) had the lowest MIC$_{90}$ (≤0.25 μg/ml) against doxycycline, levofloxacin, and clindamycin and the highest MIC$_{90}$s against chloramphenicol and linezolid (MIC$_{90}$, 8 μg/ml). Using breakpoints for S. aureus, potential resistance to linezolid was noted in three isolates (MIC, 8 μg/ml).

**Molecular analysis for genetic mechanisms of resistance.** As tetM is the most common gene associated with TET$^+$ in Ureaplasma spp. in the literature, we first conducted PCR specific to the tetM fragment in the tetracycline-resistant isolate and in four other sensitive isolates. The assay produced a bright band of ~400 bp, which aligned with U. urealyticum ATCC 33175, a type strain that harbors the tetM gene (Fig. 2). No sensitive isolates tested contained the gene.

Analysis of quinolone resistance-determining regions (QRDRs) of gyrA, gyrB, parC, and parE revealed that the levofloxacin-resistant isolate contained a C248G transversion within parC. This resulted in an S83W nonsynonymous substitution (NSS) with tryptophan encoded by TGA (Fig. 3A). Interestingly, a quinolone-sensitive U. urealyticum isolate contained two mutations within the parE QRDR: the first was a G1310T transversion, which led to an R437I NSS, and the second was a G1438A transition, which caused a G480S NSS (Fig. 3B). This isolate had a sensitive levofloxacin MIC (1 μg/ml) and an MIC to ciprofloxacin (2 μg/ml) that was not out of range compared to the other Ureaplasma species isolates tested (MIC$_{90}$, 2 μg/ml). Thus, although this mutation falls within the QRDR of parE, it appears not to impair quinolone effectiveness. No other mutations were found in any of the sequenced isolates within the QRDRs of either gyrA or gyrB.

**DISCUSSION**

UTIs affect college-aged women more so than any other population (4), and no previous studies have evaluated antibiotic resistance within this population or solely among urinary Ureaplasma species or M. hominis isolates. Further, only a few studies have characterized antibiotic resistance levels within Ureaplasma spp. within the United States. With growing levels of antibiotic resistance and a lack of data describing antibiotic resistance within this population in the United States, our study constructed an antibiogram against a panel of antibiotics capable of treating Ureaplasma species and M. hominis clinical isolates.

Overall, we found low levels of antibiotic resistance in this population of college-aged women with first-time UTIs, and to our knowledge, we identified the first levofloxacin-resistant isolate in the United States with an S83W mutation in parC. The study also provided evidence against R437I and G480S substitutions in parE causing levofloxacin resistance (LVXR). Although the overall highest MICs were associated with U. urealyticum isolates, the two clinical isolates with known resistance determinants (tetM mutation and S83W mutation in parC) were found in U. parvum isolates. In contrast, M. hominis isolates were sensitive to established breakpoints or, with the exception of three isolates that had elevated MICs to linezolid, had lower MICs than the breakpoints established for S. aureus.

This study’s strengths include the use of validated microbroth and agar dilution methods and differentiation of Ureaplasma species isolates. Studies elsewhere have used different kits to investigate antibiotic resistance in Ureaplasma spp.; however, some of these kits provided breakpoints to antibiotics that either have not been
FIG 1 Box plots comparing MICs for each antibiotic tested between *Ureaplasma urealyticum* and *U. parvum* isolates. Lower and upper whiskers on box plots represent 10 and 90th percentiles, respectively, and black circles represent outliers. *U. urealyticum* had significantly higher MICs than *U. parvum* isolates against all antibiotics except for doxycycline (not pictured).
properly evaluated or do not coincide with the CLSI standards (22). Further, some kits, such as the Mycoplasma IST2 (MIST2) kit, verify if a minimum inoculum is achieved but do not identify if levels exceed 10⁵ CFU/ml, an inoculum level proven to give false-positive results (23). Besides the two previous concerns, a separate study (24) evaluating the MIST2 kit in a subset of samples found that 75% of isolates had MICs per broth microdilution methods within 1 or 2 dilutions below the 4 μg/ml ciprofloxacin resistance cutoff designated by MIST2. However, the MIST2 assay identified all samples as resistant (24). These data call into question the validity of the kit and may lead to inaccurate reports of elevated antibiotic resistance levels. Fortunately, all U.S. studies conducted have used broth microdilution methods, allowing for direct MIC comparisons. A study limitation includes sample collection methodology. Study participants provided a midstream clean-catch urine specimen, but despite the use of this methodology, vaginal microflora could have contaminated the urinary specimens, possibly providing cultures reflecting genital tract versus the urinary biota.

Although we detected only one levofloxacin-resistant isolate, it did have an NSS identified only twice previously in Ureaplasma species clinical isolates (25, 26). The first report of this mutation surfaced in a Japanese isolate (25); unfortunately, the authors were unable to cultivate the isolate for MIC testing. However, in silico modeling indicated that an S83W substitution in parC caused steric hindrance within the protein, which prevented levofloxacin from binding to its target (25). A 2015 study performed in China uncovered the first phenotypic evidence of LVXr associated with an S83W NSS in a U. parvum isolate. Their corresponding MICs (levofloxacin, 4 μg/ml; ciprofloxacin, 16 μg/ml) matched those of our levofloxacin-resistant isolate (26). Recent studies in the

### TABLE 2 Summary of MIC results for 10 Mycoplasma hominis clinical isolates

<table>
<thead>
<tr>
<th>Drug used</th>
<th>MIC data (μg/ml)</th>
<th>No. (%) of resistant isolatesa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC₉₀</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2–8</td>
<td>4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤0.25–1</td>
<td>0.5</td>
</tr>
<tr>
<td>Clindamycinb</td>
<td>≤0.23</td>
<td>≤0.23</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Gentamicinc</td>
<td>2.6–10.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>≤0.25–0.5</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1–8</td>
<td>4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≤0.25–1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

- aResistance determined by using CLSI interpretive guidelines. NA, not applicable, as there are no established breakpoints. Breakpoints: levofloxacin, ≥2 μg/ml; clindamycin, ≥0.5 μg/ml; and tetracycline, ≥8 μg/ml.
- bEnd MICs adjusted to account for drug potency.

![FIG 2](https://journals.asm.org/journal/aac) PCR results screening for tetM on 2% agarose gel. Lane 1, GeneRuler low-range DNA ladder (MW, molecular weight in base pairs); lane 2, amplicon from U. urealyticum ATCC 33175, which harbors the tetM gene; lane 3, amplicon from the tetracycline-resistant clinical isolate 36; lanes 4 to 7, amplicon from tetracycline-sensitive Ureaplasma species clinical isolates; lanes 8 and 9, amplicon from U. parvum ATCC 27815 and U. urealyticum ATCC 27618; lane 10, negative control. The tetM expected gene product is 397 bp based on the primers used.
United States, Japan, and England and a review cataloguing mutations in *Ureaplasma* species isolates from 2006 to 2016 found that the S83L substitution in *parC* solely accounted for LVXr in the majority of isolates (range, 55 to 87%) examined (17, 19, 24, 25, 27). Transversions are less likely seen in nature due to the disruptiveness of substituting a purine for a pyrimidine or vice versa (28). This may explain the scarcity of seeing an S83W *parC* substitution.

The equivalent of a *parC* S83W substitution has been identified in at least six studies investigating mechanisms of quinolone resistance in clinical isolates of *Acinetobacter baumannii* (29), extended-spectrum beta-lactamase-producing (ESBL) *E. coli* (30), and *Pseudomonas aeruginosa* (31–34). In *P. aeruginosa* isolates, this substitution was detected in 6% of samples in three studies (31–33); however, Pasca et al. (34) identified the substitution in 16 (35%) *P. aeruginosa* isolates. In the studies evaluating ESBL *E. coli* and *A. baumannii* samples, the NSS appeared in 3% of isolates (29, 30). Each isolate containing the S83W substitution also had a coexisting NSS within the QRDR of *gyrA*.

Therefore, it appears that the S83W substitution in *parC* also occurs infrequently in other bacterial species.

In comparison to studies performed in the United States, antibiotic resistance levels in this population were generally lower. A study in Minnesota (19) found slightly lower TETr among their isolates (0.4%) than in our study (1.4%); however, two previous studies in the Southeast United States found TETr levels in 33 and 34% of isolates (17, 18). Similarly, this study found lower levels of LVXr in this population (1.4%) than in other U.S. studies (5 to 6%) (17, 19). Lower antibiotic resistance levels may be attributed to three factors related to our study population. First, our population’s younger age generally indicates a healthier population. Thus, they are less likely to have comorbidities that would increase the likelihood of hospitalization or exposure to antibiotic-related risk factors for antibiotic-resistant UTIs (35, 36). Second, the women in our study

**FIG 3** Amino acid alignment of the *parC* QRDR (A) and *parE* QRDR (B) for *U. parvum* ATCC 27815 (#1), a sensitive *U. parvum* clinical isolate (#2), the levofloxacin-resistant isolate (#3), *U. urealyticum* ATCC 33699 (#4), and a sensitive *U. urealyticum* clinical isolate (#5). The red box highlights the nonsynonymous substitution (NSS) within the levofloxacin-resistant isolate in panel A, the orange box shows the neutral NSS in panel B, and the green boxes in both panels identify normal polymorphisms previously established between *Ureaplasma* species (27). The *parC* QRDR (shown as 1 to 104) corresponds to amino acids 50 to 153 in the ParC protein, encoded by nucleotides 149 to 457 in the *parC* gene. The *parE* QRDR (shown as 1 to 105) corresponds to amino acids 404 to 508 in the ParE protein, encoded by nucleotides 1210 to 1524 in the *parE* gene. This amino acid alignment was generated using the Geneious alignment tool with the default options. ser., serovar.
had uncomplicated first-time UTIs. A previous study conducted at a student health care center in North Carolina found ciprofloxacin resistance in 11.8% of E. coli isolates from women with a previous UTI but only in 1.8% of isolates from women with no prior UTI (37). Thus, women experiencing a second UTI probably have had past antibiotic exposure, which increases the selective pressure that may give rise to antibiotic resistance. A third possibility for lower LVXr in our study could stem from antibiotic prescribing patterns among physicians. A study that analyzed antibiotic prescribing trends from 2002 to 2011 in U.S. women with uncomplicated UTI identified fluoroquinolones as the most common antibiotic prescribed. However, each age group over 30 had significantly higher odds of receiving fluoroquinolones for treatment than those in women age 18 to 29 (38). Since other U.S. studies included isolates from a variety of patients that encompassed a broader age range, their population may have had higher fluoroquinolone exposure, further enhancing selective pressures that promote levofloxacin-resistant organisms.

As seen in two previous studies conducted in England and the United States, this study in general echoes the higher MIC trend that U. urealyticum exhibits compared to U. parvum, regardless of antibiotic resistance differences between species (19, 24). This was the case in our study for all antibiotics tested, except for doxycycline. In the English study, U. urealyticum proved to have significantly higher MICs for tetracycline, ciprofloxacin, erythromycin, and gentamicin. However, they did not find significantly different MICs between species to azithromycin and chloramphenicol (24). Similar to our findings, a separate U.S. study found that MIC50s for U. urealyticum were one dilution higher for levofloxacin, ciprofloxacin, tetracycline, erythromycin, and azithromycin, but not for doxycycline, basically in line with our findings (19).

In conclusion, this study provides the first data on antibiotic resistance levels in Ureaplasma spp. within college-aged women with first-time UTIs, as well as the first molecular and phenotypic evidence of LVXr caused by a parC S83W mutation in the United States. Although the study detected low levels of antibiotic resistance, clinician researchers should consider incorporating periodic surveillance for antibiotic resistance in mycoplasmas. As elevated levels of TETr exist regionally, and sexual transmission serves as the primary transmission pathway for Ureaplasma spp. and M. hominis in adults (9), strains harboring this gene could easily spread. Thus, periodic surveillance can alert providers to changes in regional antibiotic resistance patterns so they can adjust their empirical treatment selection accordingly to choose the most effective treatment.

MATERIALS AND METHODS

Study description. Isolates from this study were collected from women enrolled in a previously conducted prospective cohort study designed to identify epidemiological risk factors associated with first-time UTI in college-aged women (20). Briefly, between 2001 and 2006 at a student health care center in Florida, the study enrolled 185 women with first-time UTI, defined as having a urinalysis positive for leukocyte esterase and a corresponding UTI symptom(s), along with 80 age-matched controls. Microbial urine cultures were performed at the initial clinical presentation and for any recurrent UTI episode. The University of Florida institutional review board approved the original study, and subsequent approval was obtained for this secondary analysis.

Clinical isolates and species determination of Ureaplasma species. Urine samples were processed for culture, as previously described (20). A total of 73 Ureaplasma species and 10 M. hominis isolates were obtained from the urine of women with initial or recurrent UTI. We used PCR to differentiate U. parvum and U. urealyticum using primers adapted from a study by Robertson et al. (39). The assay consists of two separate PCRs, each using a primer that anneals to a conserved region (P6), and a primer specific to each species (UP-3 and UU-8; Table S1). A 1-ml overnight culture was centrifuged at 14,000 rpm at 4°C for 1 h, followed by the addition of 20 μl of lysis buffer (25 mM Tris, 0.0006% Tween 20, 1.6 mM dithiothreitol) to cell pellets and incubation at 95°C for 20 min. PCR was performed on 5 μl of lysis and 45 μl of master mix containing a final concentration of 1 × Colorless GoTaq Flexi buffer, 1.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.25 μM each forward and reverse primer, and 1.25 units of GoTaq DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1. Both UP3-16S-F and P6-16S-R primers were modified by one nucleotide to match DNA polymerase, sourced from Promega (Madison, WI). The primers and annealing temperatures are provided in Table S1.
(1 min at primer-specific temperature), and elongation (72°C for 1 min/kb), and a final extension cycle for 10 min at 72°C.

**MIC determination.** We used previously validated microbroth and agar dilution methods (21) to determine the MICs for a panel of antibiotics. Ciprofloxacin, gentamicin, erythromycin, doxycycline, and levofloxacin powders were purchased from Sigma-Aldrich (St. Louis, MO, USA), and chloramphenicol powder was purchased through AmericanBio (Natick, MA, USA). Azithromycin, clindamycin, and linezolid were provided from Pfizer through their Compound Transfer Program. Antibiotics were stored as per the manufacturer’s recommendations and dissolved and diluted according to CLSI guidelines (22). As gentamicin and clindamycin were acquired in a salt form, the study adjusted the end MICs for each organism and drug to account for potency. *M. hominis* is intrinsically resistant to azithromycin and erythromycin (40) and *Ureaplasma* is intrinsically resistant to linezolid (41).

The broth microdilution assay involved setting up a sterile 96-well plate(s) for each clinical isolate of *Ureaplasma*, with each antibiotic tested in duplicate in the range of 64 μg/ml to 0.25 μg/ml. Columns 9 to 12 served as the growth, solvent, medium, and drug controls, respectively. Wells 1 to 9 received 175 μl of *Ureaplasma* spp. and were preincubated for 1 h in fresh growth medium to yield 104 to 105 CFU/ml. Following inoculation, plates were secured with sterile acetate sealers, incubated at 37°C in ambient air, and evaluated when the growth control turned pink, usually between 10 and 16 h. The MIC was interpreted as the lowest concentration of antibiotic that inhibited any color change along the spectrum from yellow to pink. *Ureaplasma* ATCC 33175 was incorporated to ensure that the type strain MIC fell within acceptable previously established quality control ranges (40). The assay used batches of laboratory-prepared 10B broth and A8 agar for MIC determination and for CFU confirmation, with a pH ranging between 5.8 and 6.0.

Because *M. hominis* does not demonstrate distinct color changes during growth, the MICs for 10 *M. hominis* isolates were determined using a previously validated agar dilution method (21). Briefly, 2 ml of either a specific antibiotic concentration (range, 64 μg/ml to 0.25 μg/ml per antibiotic tested) or a 1:10 solution of 95% ethanol (solvent control plate) was added to 18 ml of molten SP4 agar and dispensed into a square petri dish. Using a Steer’s replicator, 10 μl of each organism at concentrations of 106, 105, and 104 CFU/ml were added to each antibiotic dose plate, solvent, and growth control plates. Using the dilution that produced a concentration between 104 and 105 CFU/ml, the MIC for the isolate was interpreted as the lowest antibiotic concentration that inhibited colony formation at the time when the growth control plate exhibited colony growth. The assay also evaluated the MIC of *M. hominis* ATCC 23114 against each antibiotic to ensure quality control. Laboratory batches of SP4 broth and agar with 21% arginine were used to conduct the assays (pH range, 7.0 to 7.2).

**Evaluating molecular determinants of resistance.** PCR was used to screen for tetM, the most common gene associated with TET in *Ureaplasma* spp. (9, 23). *Ureaplasma* ATCC 33175, which harbors the tetM gene, served as a positive control, while *U. urealyticum* ATCC 27618, *U. parvum* ATCC 27815, and four randomly selected tetracycline-sensitive clinical isolates served as negative controls. A major determinant of quinolone resistance in *Ureaplasma* spp. is point mutations causing NSS in either of the DNA gyrase genes (*gyrA* and *gyrB*) and/or topoisomerase IV genes (*parC* and *parE*). We used previously published primers (17, 27, 42) to amplify the QRDRs of these four genes. The same PCR master mix final concentrations as described above for *Ureaplasma* species determination PCR were used (39), with the following exceptions: the master mix reagent concentration varied in that the longer amplicon required a higher concentration of MgCl₂ (2.5 mM), dNTPs (0.3 mM each), primers (0.4 μM), and GoTaq DNA polymerase (2 units/reaction). The thermocycler conditions used for amplification of QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were identical to those used for *Ureaplasma* species determination. The primers and annealing temperatures used are provided in Table S1.

For sequencing of the QRDR regions of the DNA gyrase genes (*gyrA* and *gyrB*) and topoisomerase IV genes (*parC* and *parE*), we chose the only levofloxacin-resistant isolate and two randomly selected sensitive control isolates. QRDR PCR products were purified using the QiAquick PCR purification kit (Qiagen, Germantown, MD, USA), according to the manufacturer’s protocol, and sent for Sanger sequencing (Lone Star Labs, Inc., Houston, TX, USA). Additional sequencing primers were designed for *gyrA* and *parE* (Table S1). Bioinformatic analysis of the Sanger sequencing results was conducted using Geneious version 10.0.08. Trace files were trimmed with a 0.05 error probability limit, followed by de novo assembly using the Geneious assembler to create a consensus sequence, using the default options. Under the consensus sequence settings, “highest quality” was selected, which constructs a majority consensus sequence by considering relative base quality. Consensus sequences for the *gyrA*, *gyrB*, *parC*, and *parE* genes for each isolate under analysis were mapped to its corresponding species, with *U. urealyticum* ATCC 27618 and *U. parvum* ATCC 27815 serving as reference strains for either species. The gene sequences for each type strain were retrieved from GenBank.

**Statistical analyses.** Differences in MICs between *Ureaplasma* species using isolates collected at initial and recurrent time points were determined by the Wilcoxon-Mann-Whitney test. Statistical analyses were conducted using Prism for Windows version 7.03. A *P* value of <0.05 was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01104-17.

**SUPPLEMENTAL FILE 1,** PDF file, 0.1 MB.
ACKNOWLEDGMENTS

Archival samples were obtained from a study funded by NIAID grant R01 AI45875 (to M.B.B.). Azithromycin, clindamycin, and linezolid were provided by Pfizer through their Compound Transfer Program. M.A.V.-K. is supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under University of Florida Clinical and Translational Science awards TL1TR001428 and UL1TR001427.

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

REFERENCES


42. Bebear CM, Renaudin H, Charon A, Gruson D, Lefrancois M, Bebear C. 2000. In vitro activity of trovafloxacin compared to those of five antimicrobials against mycoplasmas including *Mycoplasma hominis* and *Urea-