Antibiotic Resistance among Clinical *Ureaplasma* Isolates from Cuban individuals between 2013 and 2018.

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Abstract

Introduction. Acquired resistance against the antibiotics that are active against *Ureaplasma* species have been described, and diagnostics combined with antimicrobial sensitivity testing are required for therapeutic guidance.

Aim. To report the prevalence of antimicrobial resistance among Cuban *Ureaplasma* isolates and the related molecular mechanisms of resistance.

Methodology. Traditional broth microdilution assays were used to determine antimicrobial sensitivity testing in 262 clinical *Ureaplasma* species isolates from Cuban patients, between 2013 and 2018, and a subset of samples were investigated in parallel with the commercial MYCO WELL D-ONE rapid culture diagnostic assay. The underlying molecular mechanisms for resistance was determined by PCR and sequencing for all resistant isolates.

Results. Among the tested isolates, the tetracycline and erythromycin resistance rates were 1.9% and 1.5% respectively, while fluoroquinolone resistance was not found. The *tet*(M) gene was found in all tetracycline-resistant isolates, but also in two tetracycline-susceptible *Ureaplasma c*linical isolates. No mutations were found in the erythromycin resistance isolates. The MYCO WELL D-ONE kit overestimated tetracycline and erythromycin resistance in *Ureaplasma* spp. isolates.

Conclusions. Although low levels of antibiotic resistance were detected in Cuban patients over a 5-year period, continued surveillance of the antibiotic susceptibility of *Ureaplasma* is necessary to monitor possible changes in resistance patterns.

INTRODUCTION.

Ureaplasma species are the most prevalent genital mycoplasma isolated from the urogenital tract of both men and women, and are gaining recognition as pathogens in adult and neonatal patient groups. In adults, *Ureaplasma* spp. has been linked with nongonococcal urethritis, cervicitis, and pelvic inflammatory disease (1). Associations with adverse pregnancy outcomes, including miscarriage, chorioamnionitis, and preterm birth, as well as chronic lung disease, bacteremia and meningitis in newborns have also been suggested (2).

The absence of a bacterial cell wall renders *Ureaplasma* spp. intrinsically resistant to all beta-lactam and glycopeptide antibiotics. The three classes of antibiotics which are recognized as active against *Ureaplasma* spp. are the quinolones, tetracyclines, and macrolides (3). Mutations in one or both of the two copies of 23S rRNA in the genome or, more frequently, amino acid substitutions in the L4 and L22 ribosomal proteins were linked previously to macrolide resistance. Accumulation of point mutations in the quinolone resistance-determining regions of the parC gene are the predominant mechanisms of resistance to macrolides and fluoroquinolones, while acquisition of the gene encoding the Tet(M) ribosomal protection protein on the Tn916-like mobile element being associated with resistance to tetracycline (4). Various studies on the antimicrobial susceptibility profiles and resistance mechanisms of genital mycoplasmas have been found to vary widely over different geographic regions (5-7).

In this report, we describe the prevalence of antimicrobial resistance among *Ureaplasma* isolates from Cuban patients and the related molecular mechanisms of resistance.

METHODS.

Clinical samples.

A total of 262 clinical *Ureaplasma* species isolates from patient samples, submitted for mycoplasma diagnostic testing in the National Reference Laboratory of Mycoplasmas, at the Tropical Medicine Institute “Pedro Kouri” between 2013 and 2018, were examined. The *Ureaplasma* species was determined by qPCR targeting of species specific polymorphisms in the *ureC* gene as previously described (8). The sample source comprised a variety of patient groups: 13 cervical samples from pregnant women, 130 from women with leucorrhea, 43 from women under investigation for infertility antecedents, 29 women who had spontaneous abortion, 3 neonatal respiratory samples from ventilated newborns and 44 urine samples from men with non-gonococcal, non-chlamydia urethritis.

Determination of antibiotic susceptibility with the broth microdilution method and MYCO WELL D-One assay.

Minimal inhibitory concentration (MIC) were determined for 42 clinical isolates, identified initially by molecular diagnostics during the period of 2013 to 2016**,** and recovered from archives for antimicrobial sensitivity testing (AST) as previously described by Beeton *et al*. (9), adhering to the Clinical and Laboratory Standards Institute (CLSI) guidelines (10). MICs were determined for the antibiotics tetracycline, levofloxacin, moxifloxacin and erythromycin in a range of 0.06 μg ml^{-1} to 64 μg ml^{-1}. The antibiotics were obtained from Sigma-Aldrich (Dorset, United Kingdom), and *Ureaplasma* selective medium (Mycoplasma Experience, Reigate, UK) was used for the microdilution broth assay.

Two hundred and twenty one isolates were identified by screening with the MYCO WELL D- One commercial kit (CPM Scientifica, Italy) from 810 clinical samples tested during 2016 to 2018, and antibiotic susceptibility testing results were interpreted according to the manufacturer's instructions. When resistance to any of the antibiotics was detected, broth microdilution MICs were systematically determined for confirmation for tetracycline, levofloxacin, moxifloxacin and erythromycin by broth microdilution using *Ureaplasma* Shepard medium (*in house*). Additionally, 12 randomly chosen isolates identified as susceptible by the commercial kit were also analyzed by traditional AST for susceptibility confirmation.

Since the MYCO WELL D-ONE kit cannot distinguish between *U. urealyticum* and *U. parvum* species, post-identification speciation was performed by qPCR as above (8).

PCR and sequencing of resistance genes.

Bacterial DNA extraction from broth culture of resistant isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany). PCR and sequencing of the domain II and V of the 23S rRNA of erythromycin resistant isolates, determined by broth microdilution, as well as amplification and sequencing of conserved portions of L4 and L22 genes was carried out using primers previously described (11). The sequences were analysed using the Geneious software (version R10, Biomatters ltd, New Zealand), and compared to reference strains *U. parvum* serotype 3 (ATCC 27815) and *U. urealyticum* serotype 8 (ATCC 27618) (GenBank accession numbers NC_010503.1 and NZ_AAYN02000002.1, respectively)

The presence of the *tet*(M) gene in the tetracycline-resistant strains identified by MIC was confirmed by qPCR using primers tetM1378R (GCATTCCACTTCCCAACGGA) and tetM1309F (GTGCCGCCAAATCCTTTCTG) and probe tetMqPCR1309F (Cy5-CCATTGGTTTATCTGTATCACCGC-BHQ3) to amplify a 70 bp fragment, melting temperature[Tm] of 60°C and 35 cycles. These primers and probe were designed against in conserved elements for *tet*(M) gene containing strains subjected to whole genome sequencing in house.

Statistical analysis.

The x^2 test and Fisher's exact test were used to compare the occurrence of resistant isolates that were identified using commercial kits with the occurrence of resistant isolates identified using MICs.

RESULTS.

Prevalence of resistance.

Using CLSI-compliant broth microdilution technique for the 42 isolates obtained by culture, we were able to identify 2 tetracycline-resistant *U. parvum* isolates (MICs of 4 $\mu g\ ml^{-1}$ and 16 $\mu g\ ml^{-1}$) and 2 *U. urealyticum* and 1 *U. parvum* erythromycin-resistant isolates (MICs of 16 $\mu g\ ml^{-1}$, 32 $\mu g\ ml^{-1}$and 64 $\mu g\ ml^{-1}$ respectively). All the isolates were sensitive to levofloxacin and moxifloxacin.

Evaluation of *Ureaplasma* spp. resistance using MYCO WELL D-ONE commercial kit and MIC determinations.

Of the 220 *Ureaplasma* spp. isolates analyzed by MYCO WELL D-One kit, 3.6% (8/220) were identified as being levofloxacin-resistant (MIC ≥4 μg ml^{-1}), 1.4% (3/220) as being moxifloxacin-resistant (MIC ≥ 4 μg ml^{-1}), 15.9% (35/220) as being tetracycline-resistant (MIC ≥ 2 μg ml^{-1}) and 17.3% (38/220) as being erythromycin-resistant (MIC ≥ 16 μg ml^{-1}) isolates.

When MICs were determined for these isolates, only 3/35 were confirmed as resistant to tetracycline, 1/38 confirmed as resistant to erythromycin and none were confirmed resistant to levofloxacin nor to moxifloxacin (0/8 and 0/3 respectively). One dual-resistant strain to tetracycline and erythromycin obtained by the commercial kit was confirmed by MIC determination. Additionally, the 12 randomly chosen isolates shown by the commercial kit to be susceptible were confirmed as susceptible by accurate MIC determination.

Overall, within the study period of 2013-2018, 1.9 % (5/262) of isolates were found to be resistant to tetracycline and 1.5% (4/262) were found to be resistant to erythromycin. Table1.

Table1. Overview of antibiotic-resistant isolates identified from Cuba samples between 2013 and 2018.

late (year lated)	**Type of sample/patient**	**Species of *Ureaplasma***	**Antibiotic resistance (MIC in μg ml^{-1})**	**Mechanism of resistance**
681(2013)	Cervical/woman	*U. parvum*	Tetracycline (16)	*tet* (M) positive
211(2014)	Cervical/woman	*U. parvum*	Tetracycline (4)	*tet* (M) positive
188(2016)	Cervical/woman	*U. parvum*	Tetracycline (64) Erythromycin (16)	*tet* (M) positive
189(2016)	Cervical/woman	*U. urealyticum*	Erythromycin (32)	N.D.*
192(2016)	Cervical/woman	*U. urealyticum*	Erythromycin (64)	N. D.*
106(2017)	Respiratory/neonate	*U. parvum*	Erythromycin (16)	N. D.*
296(2017)	Cervical/woman	*U. parvum*	Tetracycline (32)	*tet* (M) positive
593(2017)	Cervical/woman	*U. parvum*	Tetracycline (32)	*tet* (M) positive

**N* D.*: not determined*

Molecular mechanism for erythromycin resistance.

166 The underlying molecular mechanism for resistance in the 4 erythromycin-
167 resistant isolates (confirmed by MIC determination) was analyzed by
168 sequencing key genes. Sanger sequencing of L4 and L22 genes amplified by
169 PCR showed substitution for these isolates but no deletions nor any significant
170 changes to amino acid sequence. Sanger sequencing of the domain V of the
171 23S rRNA for each independent copy of the 2 operons in the *Ureaplasma*
172 genome did not reveal any mutations associated to macrolide resistance.
173
174 **Screening for tetracycline resistance gene.**
175 All tetracycline-resistance isolates identified by both methods were screening by
176 qPCR for the presence of the *tet*(M) gene. However, the 35 isolates that failed
177 to be confirmed by accurate MIC determination above were negative for the
178 *tet*(M) gene. Only, the 5 isolates confirmed by broth microdilution method to
179 have an MIC >2 $\mu g\ ml^{-1}$for tetracycline were found to be positive for the *tet*(M)
180 gene. In addition, 2 of the susceptible isolates by the kit and confirmed by MIC
181 determination were positive to *tet(*M) gene.
182 **DISCUSSION**
183 In recent years, *Ureaplasma* spp. have received increased attention because of
184 their association with numerous clinical presentations. The limited therapeutic
185 options available to combat infections caused by this urogenital mycoplasma
186 justify the importance of studying the prevalence and mechanisms of resistance
187 (12). However, according to the methodology of detection, resistance data may
188 not be comparable. Commercial kits are an easy method for initial screening,
189 but indication of resistance needs to be followed up appropriately, not just
190 reported, as recommended in the recent literature (13). Particularly as only a

very low minority of the commercial kits set their screening levels in line with the internationally set CLSI breakpoints for resistance, such as the MYCO WELL D-ONE kit.

In the present study, we determined the prevalence of resistance of a large number of *Ureaplasma* isolates, obtaining a low percentage of resistance to tetracycline and erythromycin, 1.9% and 1.5% respectively. This is the first study conducted in Cuba using conventional methods for the detection of resistance in ureaplasmas and the molecular characterization of the resistance. Previous studies published by Diaz *et al.* and Rodriguez *et al.* reported high percentages of antimicrobial resistance in *Ureaplasma*-positive samples detected by commercial kits (14, 15), but no confirmation of resistance by conventional or molecular methods were performed.

Similar results of resistance were found by Valentine-King *et al.* and Fernandez *et al* in USA, who report 1.4% and 0.4% of tetracycline-resistant isolates respectively, obtained from diverse samples sources of college-aged females, neonates and adults, and macrolide resistance was not found in these studies (16, 17). Beeton *et al* examined the prevalence of antimicrobial resistance in England and Wales in clinical Ureaplasma isolates from women and neonates, and identified 2.3% tetracycline resistance prevalence and an absence of resistance to macrolides (18). Higher percentages of tetracycline resistance have been documented by Meygret *et al* who analyzed a higher number and types of clinical samples in their study (12).

Tetracycline resistance is well characterized among *Ureaplasma* and mediated via the acquisition of the *tet*(M) resistance element, giving ribosomal protection (19). All tetracycline- resistant strains in this study were positive for *tet*(M) in

addition to 2 tetracycline-sensitive isolates from a small number of the susceptible isolates. The presence of *tet*(M) in tetracycline-susceptible isolates had been previously documented (9, 18, 19). Some *tet*(M) variants may exhibit inducible resistance, and therefore it may be necessary to screen by both broth microdilution to assess phenotypic susceptibility and molecular methods to detect *tet*(M) variants (18).

The mechanism of macrolide resistance in clinical *Ureaplasma* spp. is less well characterized, since macrolide-resistant *Ureaplasma* spp. are uncommon at the international level (4). Govender *et al* found 26.7 % of erythromycin resistant isolates in pregnant women, with L22 ribosomal proteins alterations associated to resistance (20). Xiao *et al* found 1% of erythromycin-resistant clinical isolates obtained from variety of clinical specimens, with point mutations in the 23S rRNA in addition to L22 and L4 ribosomal protein substitutions associated to resistance (21). More recently, Yang *et al* found 3.59 % of erythromycin-resistant clinical isolates obtained from urogenital tract specimens, but couldn´t identified mutations neither in the ribosomal proteins or the 23S rRNA related to macrolide resistance (22).

In our study the clinical isolates tested did not reveal any mutations in the region genes analyzed that could be related to macrolide resistance. Other resistance mechanisms described in bacteria included drug inactivation, active drug efflux pumps, and modification of the target site by methylation. Lu Ch *et al* found *U. urealyticum* clinical isolates carried the *erm*B methylase gene and *msr* genes, one of the common active efflux genes that confers low level resistance to 14- and 15-membered macrolides (23). Yang T *et al* reaffirm the find of the *ermB*

gene in one *U. parvum* macrolide resistant isolate (22). We didn´t search for this genes in the present study.

In this study, we found that MYCO WELL D-ONE kit overestimated antimicrobial resistance in *Ureaplasma* spp. isolates. Studies elsewhere have used different commercial kits to investigate antibiotic resistance in *Ureaplasma* spp.; however, as previously highlighted, most of these kits examine antibiotic concentrations that are below the internationally agreed breakpoints defined for true resistance as set by the CLSI standards. Schneider *et al* found conflicting results from the Mycoplasma IST2 kit and standard broth microdilution for ciprofloxacin and azithromycin, where most of the isolates routinely reported as nonsusceptible to these antibiotics were actually fully sensitive (1). Piccinelli *et al* also demonstrated that the Mycoplasma IST2 kit overestimated the fluoroquinolone resistance giving false resistance results when compared to the microdilution method. However, this is expected as the Mycoplasma IST2 kit utilizes 2mg/L as the cut-off for ciprofloxacin, which is less effective than levofloxacin and well below the 4mg/L breakpoint internationally agreed (24, 9). A recent study using another commercial kit, the MYCOFAST RevolutioN kit, which does utilize the CLSI breakpoints defined for *Ureaplasma*, was also found to overestimate fluoroquinolone resistance in *Ureaplasma* spp. isolates. This underscores the fact that all commercial assays (even those that comply with CLSI antibiotic breakpoints) used in routine diagnosis, should be confirmed with broth microdilution assays according to CLSI guidelines or with molecular screening methods that detect mechanisms of resistance (12).

Unlike most commercial assays available for screening, the MYCO WELL D-One kit utilizes the CLSI breakpoints but also is unique in the examination of

Ureaplasma spp. and *M. hominis* infections separately. This is another common cause for incorrect antimicrobial resistance reporting, as *M. hominis* is inherently resistant to macrolides; therefore, it is impossible for kits such as the Mycoplasma IST2 and MYCOFAST RevolutioN to identify erythromycin resistance in a mixed infection (which does occur in 5-60% of the samples depending on the group examined) (7, 25). However, the MYCO WELL D-ONE was also found to overestimate the antimicrobial resistance, especially for tetracycline, and perhaps the reason arises from the fact that none of these kits uses a dilution method to accurately quantify the inoculum that is added to the test panel (26). The CLSI guidelines also control for bacterial input as it is well established that a load $>10^5$ cfu/ml will give a false-resistant result (10), likely because the readout is determined by pH change and urease concentration at high bacterial loads in samples (even if the protein synthesis is completely inhibited) is sufficient to change the medium color to red.

In conclusion, while these assays are immensely useful in screening of large populations or in conditions where laboratory support is poor; confirmation by traditional methods for any positive sample, to ensure the inoculum tested is approximately 10^4 cfu/ml, would ensure that the results are reliable (13). In the other hand, although this study detected low levels of antibiotic resistance in Cuban patients over a 5-year period, clinician researchers should consider incorporating periodic surveillance for antimicrobial resistance in mycoplasmas. Given that sexual transmission serves as the primary transmission pathway for *Ureaplasma* spp. and other mycoplasma species in adults, and elevated levels of tetracycline and other drugs resistance exists regionally, strains harboring this gene could easily spread. Thus, changes in regional antibiotic resistance

patterns can occur and it may be necessary to alter first line choices for most effective treatment.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This study was approved by The “Pedro Kourí” Tropical Medicine Institute Ethical Board (approval CEI-IPK 33-12).

Funding information

This work was supported by the Global Challenges Research Fund Fellowship administered by Cardiff University.

Acknowledgements.

We would like to thank Dra. Carmen Fernández who provided excellent technical and writing assistance of the article.

Abbreviations:

AST: antimicrobial sensitivity testing

MIC: Minimal inhibitory concentration

CLSI: Clinical and Laboratory Standards Institute

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