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.

47 *Ureaplasma* species are the most prevalent genital mycoplasma isolated from
48 the urogenital tract of both men and women, and are gaining recognition as
49 pathogens in adult and neonatal patient groups. In adults, *Ureaplasma* spp.
50 has been linked with nongonococcal urethritis, cervicitis, and pelvic
51 inflammatory disease (1). Associations with adverse pregnancy outcomes,
52 including miscarriage, chorioamnionitis, and preterm birth, as well as chronic
53 lung disease, bacteremia and meningitis in newborns have also been
54 suggested (2).
55 The absence of a bacterial cell wall renders *Ureaplasma* spp. intrinsically
56 resistant to all beta-lactam and glycopeptide antibiotics. The three classes of
57 antibiotics which are recognized as active against *Ureaplasma* spp. are the
58 quinolones, tetracyclines, and macrolides (3). Mutations in one or both of the
59 two copies of 23S rRNA in the genome or, more frequently, amino acid
60 substitutions in the L4 and L22 ribosomal proteins were linked previously to
61 macrolide resistance. Accumulation of point mutations in the quinolone
62 resistance-determining regions of the parC gene are the predominant
63 mechanisms of resistance to macrolides and fluoroquinolones, while acquisition
64 of the gene encoding the Tet(M) ribosomal protection protein on the Tn916-like
65 mobile element being associated with resistance to tetracycline (4). Various
66 studies on the antimicrobial susceptibility profiles and resistance mechanisms of
67 genital mycoplasmas have been found to vary widely over different geographic
68 regions (5-7).
69 In this report, we describe the prevalence of antimicrobial resistance among
70 *Ureaplasma* isolates from Cuban patients and the related molecular
71 mechanisms of resistance.

72 **METHODS.**

73 **Clinical samples.**

74 A total of 262 clinical *Ureaplasma* species isolates from patient samples,
75 submitted for mycoplasma diagnostic testing in the National Reference
76 Laboratory of Mycoplasmas, at the Tropical Medicine Institute “Pedro Kouri”
77 between 2013 and 2018, were examined. The *Ureaplasma* species was
78 determined by qPCR targeting of species specific polymorphisms in the *ureC*
79 gene as previously described (8). The sample source comprised a variety of
80 patient groups: 13 cervical samples from pregnant women, 130 from women
81 with leucorrhea, 43 from women under investigation for infertility antecedents,
82 29 women who had spontaneous abortion, 3 neonatal respiratory samples from
83 ventilated newborns and 44 urine samples from men with non-gonococcal, non-
84 chlamydia urethritis.

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

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

97 Two hundred and twenty one isolates were identified by screening with the
98 MYCO WELL D- One commercial kit (CPM Scientifica, Italy) from 810 clinical
99 samples tested during 2016 to 2018, and antibiotic susceptibility testing results
100 were interpreted according to the manufacturer's instructions. When resistance
101 to any of the antibiotics was detected, broth microdilution MICs were
102 systematically determined for confirmation for tetracycline, levofloxacin,
103 moxifloxacin and erythromycin by broth microdilution using *Ureaplasma*
104 Shepard medium (*in house*). Additionally, 12 randomly chosen isolates
105 identified as susceptible by the commercial kit were also analyzed by traditional
106 AST for susceptibility confirmation.
107 Since the MYCO WELL D-ONE kit cannot distinguish between *U. urealyticum*
108 and *U. parvum* species, post-identification speciation was performed by qPCR
109 as above (8).
110 **PCR and sequencing of resistance genes.**
111 Bacterial DNA extraction from broth culture of resistant isolates was performed
112 using the QIAamp DNA Mini kit (Qiagen, Germany). PCR and sequencing of the
113 domain II and V of the 23S rRNA of erythromycin resistant isolates, determined
114 by broth microdilution, as well as amplification and sequencing of conserved
115 portions of L4 and L22 genes was carried out using primers previously
116 described (11). The sequences were analysed using the Geneious software
117 (version R10, Biomatters ltd, New Zealand), and compared to reference strains
118 *U. parvum* serotype 3 (ATCC 27815) and *U. urealyticum* serotype 8 (ATCC
119 27618) (GenBank accession numbers NC_010503.1 and
120 NZ_AAYN02000002.1, respectively)

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

129

130 **Statistical analysis.**

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

134

135 **RESULTS.**

136 **Prevalence of resistance.**

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

142

143 **Evaluation of *Ureaplasma* spp. resistance using MYCO WELL D-ONE**
144 **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 μg $ml^{-1}$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

191 very low minority of the commercial kits set their screening levels in line with the
192 internationally set CLSI breakpoints for resistance, such as the MYCO WELL D-
193 ONE kit.
194 In the present study, we determined the prevalence of resistance of a large
195 number of *Ureaplasma* isolates, obtaining a low percentage of resistance to
196 tetracycline and erythromycin, 1.9% and 1.5% respectively. This is the first
197 study conducted in Cuba using conventional methods for the detection of
198 resistance in ureaplasmas and the molecular characterization of the resistance.
199 Previous studies published by Diaz *et al.* and Rodriguez *et al.* reported high
200 percentages of antimicrobial resistance in *Ureaplasma*-positive samples
201 detected by commercial kits (14, 15), but no confirmation of resistance by
202 conventional or molecular methods were performed.
203 Similar results of resistance were found by Valentine-King *et al.* and Fernandez
204 *et al* in USA, who report 1.4% and 0.4% of tetracycline-resistant isolates
205 respectively, obtained from diverse samples sources of college-aged females,
206 neonates and adults, and macrolide resistance was not found in these studies
207 (16, 17). Beeton *et al* examined the prevalence of antimicrobial resistance in
208 England and Wales in clinical Ureaplasma isolates from women and neonates,
209 and identified 2.3% tetracycline resistance prevalence and an absence of
210 resistance to macrolides (18). Higher percentages of tetracycline resistance
211 have been documented by Meygret *et al* who analyzed a higher number and
212 types of clinical samples in their study (12).
213 Tetracycline resistance is well characterized among *Ureaplasma* and mediated
214 via the acquisition of the *tet*(M) resistance element, giving ribosomal protection
215 (19). All tetracycline- resistant strains in this study were positive for *tet*(M) in

216 addition to 2 tetracycline-sensitive isolates from a small number of the
217 susceptible isolates. The presence of *tet*(M) in tetracycline-susceptible isolates
218 had been previously documented (9, 18, 19). Some *tet*(M) variants may exhibit
219 inducible resistance, and therefore it may be necessary to screen by both broth
220 microdilution to assess phenotypic susceptibility and molecular methods to
221 detect *tet*(M) variants (18).
222 The mechanism of macrolide resistance in clinical *Ureaplasma* spp. is less well
223 characterized, since macrolide-resistant *Ureaplasma* spp. are uncommon at the
224 international level (4). Govender *et al* found 26.7 % of erythromycin resistant
225 isolates in pregnant women, with L22 ribosomal proteins alterations associated
226 to resistance (20). Xiao *et al* found 1% of erythromycin-resistant clinical isolates
227 obtained from variety of clinical specimens, with point mutations in the 23S
228 rRNA in addition to L22 and L4 ribosomal protein substitutions associated to
229 resistance (21). More recently, Yang *et al* found 3.59 % of erythromycin-
230 resistant clinical isolates obtained from urogenital tract specimens, but couldn´t
231 identified mutations neither in the ribosomal proteins or the 23S rRNA related to
232 macrolide resistance (22).
233 In our study the clinical isolates tested did not reveal any mutations in the region
234 genes analyzed that could be related to macrolide resistance. Other resistance
235 mechanisms described in bacteria included drug inactivation, active drug efflux
236 pumps, and modification of the target site by methylation. Lu Ch *et al* found *U.*
237 *urealyticum* clinical isolates carried the *erm*B methylase gene and *msr* genes,
238 one of the common active efflux genes that confers low level resistance to 14-
239 and 15-membered macrolides (23). Yang T *et al* reaffirm the find of the *ermB*

240 gene in one *U. parvum* macrolide resistant isolate (22). We didn´t search for this
241 genes in the present study.
242 In this study, we found that MYCO WELL D-ONE kit overestimated antimicrobial
243 resistance in *Ureaplasma* spp. isolates. Studies elsewhere have used different
244 commercial kits to investigate antibiotic resistance in *Ureaplasma* spp.;
245 however, as previously highlighted, most of these kits examine antibiotic
246 concentrations that are below the internationally agreed breakpoints defined for
247 true resistance as set by the CLSI standards. Schneider *et al* found conflicting
248 results from the Mycoplasma IST2 kit and standard broth microdilution for
249 ciprofloxacin and azithromycin, where most of the isolates routinely reported as
250 nonsusceptible to these antibiotics were actually fully sensitive (1). Piccinelli *et*
251 *al* also demonstrated that the Mycoplasma IST2 kit overestimated the
252 fluoroquinolone resistance giving false resistance results when compared to the
253 microdilution method. However, this is expected as the Mycoplasma IST2 kit
254 utilizes 2mg/L as the cut-off for ciprofloxacin, which is less effective than
255 levofloxacin and well below the 4mg/L breakpoint internationally agreed (24, 9).
256 A recent study using another commercial kit, the MYCOFAST RevolutioN kit,
257 which does utilize the CLSI breakpoints defined for *Ureaplasma*, was also found
258 to overestimate fluoroquinolone resistance in *Ureaplasma* spp. isolates. This
259 underscores the fact that all commercial assays (even those that comply with
260 CLSI antibiotic breakpoints) used in routine diagnosis, should be confirmed with
261 broth microdilution assays according to CLSI guidelines or with molecular
262 screening methods that detect mechanisms of resistance (12).
263 Unlike most commercial assays available for screening, the MYCO WELL D-
264 One kit utilizes the CLSI breakpoints but also is unique in the examination of

265 *Ureaplasma* spp. and *M. hominis* infections separately. This is another
266 common cause for incorrect antimicrobial resistance reporting, as *M. hominis* is
267 inherently resistant to macrolides; therefore, it is impossible for kits such as the
268 Mycoplasma IST2 and MYCOFAST RevolutioN to identify erythromycin
269 resistance in a mixed infection (which does occur in 5-60% of the samples
270 depending on the group examined) (7, 25). However, the MYCO WELL D-ONE
271 was also found to overestimate the antimicrobial resistance, especially for
272 tetracycline, and perhaps the reason arises from the fact that none of these kits
273 uses a dilution method to accurately quantify the inoculum that is added to the
274 test panel (26). The CLSI guidelines also control for bacterial input as it is well
275 established that a load >10^5 cfu/ml will give a false-resistant result (10), likely
276 because the readout is determined by pH change and urease concentration at
277 high bacterial loads in samples (even if the protein synthesis is completely
278 inhibited) is sufficient to change the medium color to red.
279 In conclusion, while these assays are immensely useful in screening of large
280 populations or in conditions where laboratory support is poor; confirmation by
281 traditional methods for any positive sample, to ensure the inoculum tested is
282 approximately 10^4 cfu/ml, would ensure that the results are reliable (13). In the
283 other hand, although this study detected low levels of antibiotic resistance in
284 Cuban patients over a 5-year period, clinician researchers should consider
285 incorporating periodic surveillance for antimicrobial resistance in mycoplasmas.
286 Given that sexual transmission serves as the primary transmission pathway for
287 *Ureaplasma* spp. and other mycoplasma species in adults, and elevated levels
288 of tetracycline and other drugs resistance exists regionally, strains harboring
289 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).

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321
322 **Abbreviations:**

323 AST: antimicrobial sensitivity testing

324 MIC: Minimal inhibitory concentration

325 CLSI: Clinical and Laboratory Standards Institute

326

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