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Antibiotic Resistance among Clinical *Ureaplasma* Isolates Recovered from Neonates in England and Wales between 2007 and 2013

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***Ureaplasma* spp. are associated with numerous clinical sequelae with treatment options being limited due to patient and pathogen factors. This report examines the prevalence and mechanisms of antibiotic resistance among clinical strains isolated from 95 neonates, 32 women attending a sexual health clinic, and 3 patients under investigation for immunological disorders, between 2007 and 2013 in England and Wales. MICs were determined by using broth microdilution assays, and a subset of isolates were compared using the broth microdilution method and the Mycoplasma IST2 assay. The underlying molecular mechanisms for resistance were determined for all resistant isolates. Three isolates carried the *tet*(M) tetracycline resistance gene (2.3%; confidence interval [CI], 0.49 to 6.86%); two isolates were ciprofloxacin resistant (1.5%; CI, 0.07 to 5.79%) but sensitive to levofloxacin and moxifloxacin, while no resistance was seen to any macrolides tested. The MIC values for chloramphenicol were universally low (2 μg/ml), while inherently high-level MIC values for gentamicin were seen (44 to 66 μg/ml). The Mycoplasma IST2 assay identified a number of false positives for ciprofloxacin resistance, as the method does not conform to international testing guidelines. While antibiotic resistance among *Ureaplasma* isolates remains low, continued surveillance is essential to monitor trends and threats from importation of resistant clones.**

Ureaplasma spp. are gaining recognition as pathogens in both adult and neonatal patient groups. The availability of standardized molecular detection methods has increased the capacity for identifying *Ureaplasma* isolates in pathological conditions; these isolates were previously difficult to identify by specialized culture-based methods. In adults, *Ureaplasma* spp. have been linked with nongonococcal urethritis, arthritis, meningitis, chorioamnionitis, and preterm labor, whereas in neonates links have been made with bronchopulmonary dysplasia, neonatal pneumonia, and meningitis (1–6).

Upon diagnosis of infection, treatment options are limited for a number of reasons. 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. These treatment options are further limited in situations with neonates for whom the only recognized treatment is with a macrolide due to the associated toxicity of the tetracyclines and quinolones (7).

Although two human-associated *Ureaplasma* species, *Ureaplasma urealyticum* and *Ureaplasma parvum*, have been recognized since 2002 many diagnostic laboratories still do not differentiate between the two species and report findings as *U. urealyticum* by default (8). This lack of discrimination hinders epidemiological data and has been partly accountable for the lack of understanding and potential varied pathogenicity of the two species. A recent systemic review and meta-analysis by Zhang et al. has supported the idea of *U. urealyticum* contributing to the development of nongonococcal urethritis (NGU), whereas *U. parvum* does not (9). These data suggest that *U. urealyticum* may be a true pathogen in this situation, whereas *U. parvum* represents a commensal organism.

In this report, we describe the prevalence of antibiotic resistance among *Ureaplasma* isolates from England and Wales in addition to the mechanisms of resistance. We also include susceptibility testing for *Ureaplasma* spp. against chloramphenicol and gentamicin, which do not act on the cell wall but on the ribosome as the mechanism of action.

MATERIALS AND METHODS

Clinical samples. A total of 130 clinical *Ureaplasma* species isolates from anonymized unique patient samples originally submitted for clinical diagnostic tests between 2007 and 2013 were examined (Table 1). The species of *Ureaplasma* was determined by PCR as previously described (10). The sample source comprised a variety of patient groups: 61 neonatal endotracheal samples (15 *U. urealyticum*/46 *U. parvum*) from the Public Health England reference laboratory, 32 cervical samples (5 *U. urealyticum*/27 *U. parvum*) from private sexual health patients, 18 neonatal endotracheal samples (4 *U. urealyticum*/14 *U. parvum*) from the University Hospital of Wales, 16 neonatal endotracheal samples (4 *U. urealyticum*/12 *U. parvum*) from Derriford Hospital, and 3 urine samples from patients with immune deficiencies from the University Hospital of Wales (*U. parvum*).

Determination of antibiotic resistance with the broth microdilution method and Mycoplasma IST2 assay. MICs and breakpoints were determined as previously described by Beeton et al. (11), adhering to the

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TABLE 1 Source and year of isolation for *Ureaplasma* species used for MIC determination

Source[a]	2007	2008	2009	2010	2011	2012	2013	total
PHE (PCR +)	NA[b]	NA	NA	28	47	33	60	168
PHE (PCR −)	NA	NA	NA	74	137	182	194	587
PHE (recovered for MIC)	8	19	19	10	5			61
UHW (+)	7	2		2	3	3	1	18
UHW (−)	17	6		2	2	4	9	40
Plymouth (+)					2	8	6	16
Plymouth (−)					19	20	10	49
RGH (+)					3	20	9	32
RGH (−)					6	36	15	57
Urine (+)		1	2					3
Urine (−)		3	6					9

[a] Samples were obtained from Public Health England (PHE), the University Hospital of Wales neonatal intensive care unit (UHW) or immunological outpatients (Urine), the Derriford Hospital neonatal intensive care unit (Plymouth), and the Royal Glamorgan Hospital (RGH). Not all PHE isolates were recoverable from frozen archives for MIC determination. +, positive; −, negative.
[b] NA, data not available.

TABLE 2 Overview of antibiotic-resistant isolates identified from United Kingdom samples between 2007 and 2013

Isolate (year isolated)	Species of *Ureaplasma*	Antibiotic resistance (MIC in μg/ml)	Mechanism of resistance
U6 (2009)	*U. parvum*	Ciprofloxacin (32) Levofloxacin (2) Moxifloxacin (1)	E87K in ParC
HPA116 (2013)	*U. parvum*	Ciprofloxacin (16) Levofloxacin (2) Moxifloxacin (1)	S83L in ParC
HPA111 (2008)	*U. urealyticum*	Tetracycline (64) Doxycycline (16)	*tet*(M) positive
PLY157 (2013)	*U. parvum*	Tetracycline (8) Doxycycline (8)	*tet*(M) positive
HPA71 (2007)	*U. urealyticum*	Tetracycline (64[a]) Doxycycline (16[a])	*tet*(M) positive

[a] MIC following challenge with tetracycline (initial MIC of 1 μg/ml).

Clinical and Laboratory Standards Institute guidelines (12). MICs were determined for the antibiotics tetracycline, doxycycline, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, azithromycin, clarithromycin, chloramphenicol, and gentamicin in a range of 0.06 μg/ml to 64 μg/ml. The antibiotics were purchased from Sigma-Aldrich (Dorset, United Kingdom), and *Ureaplasma* selective medium (USM) was supplied by Mycoplasma Experience Ltd. (Surrey, United Kingdom). Twenty clinical samples submitted for testing to the Public Health England laboratory were examined in parallel with standard methods with the Mycoplasma IST2 (bioMérieux, France) assay, according to the manufacturer's instructions; eight were found to be positive for *Ureaplasma* spp., and the resistance for ciprofloxacin identified for all isolates was followed up by appropriate broth microdilution methods.

PCR and sequencing of resistance genes. PCR and sequencing of the quinolone resistance-determining region (QRDR) of ciprofloxacin-resistant strains was carried out as previously described and aligned to the *U. parvum* SV3 reference genome of strain ATCC 700970 (13, 14). Confirmation of the *tet*(M) gene in the tetracycline-resistant strains was determined by PCR using the forward primer IntMtet1 located at position 309 to 328 bp and the reverse primer tet2 located at position 832 to 851 bp in the coding region (melting temperature [T_m] of 55°C, 35 cycles, amplicon of 543 bp). Extended sequencing of the *tet*(M) gene was accomplished using the tetMF-78 and tetM-R_2123 primers. All primers have been previously published (11, 15).

Statistical analysis. Statistics for the means, standard deviations, standard errors, and confidence intervals (CI) for the MIC values for *U. parvum* and *U. urealyticum* were determined using GraphPad Prism, and values for these species were compared via Student's *t* test.

Nucleotide sequence accession numbers. The sequences were submitted to GenBank under the accession number KT267561.

RESULTS

Prevalence of resistance. Using the adapted broth microdilution technique, we were able to identify two isolates resistant to ciprofloxacin (U6 at 32 μg/ml and HPA116 at 16 μg/ml), and three isolates which were tetracycline resistant (Table 2). This gave a prevalence of resistance for each antibiotic of 1.5% (CI, 0.07 to 5.79%) and 2.3% (CI, 0.49 to 6.86%), respectively. No breakpoint values for resistance of ciprofloxacin are available in the CLSI guidelines (5); however, the published breakpoints for moxifloxacin and levofloxacin indicate resistance to be ≥4 μg/ml. Strains U6 and HPA116 were both more sensitive to moxifloxacin (1 μg/ml) and levofloxacin (2 μg/ml) than to ciprofloxacin (Table 2), but these values were still higher than those for our susceptible strains (≤0.25 μg/ml for moxifloxacin and ≤0.5 μg/ml for levofloxacin) (data not shown). All 130 isolates were sensitive to the macrolide antibiotics erythromycin and azithromycin as well as to chloramphenicol. All strains had intrinsically high MICs for gentamicin (MIC_{90} values of 64 μg/ml for *U. parvum* and 128 μg/ml for *U. urealyticum*). No coresistant strains were identified. The mean MICs of all antibiotics were significantly higher for *U. urealyticum* than for *U. parvum* with the exception of those for chloramphenicol and azithromycin (Table 3).

Screening for tetracycline resistance gene. Tetracycline resistance is well characterized among *Ureaplasma* species and is associated with the presence of the horizontally acquired *tet*(M) resistance gene. We screened DNA isolated from all 130 isolates by PCR for the presence of the *tet*(M) gene and identified 3 positive strains (Table 4). Interestingly, broth culture screening for tetracycline resistance only identified two of these isolates (HPA111 with an MIC of 64 μg/ml and Ply157 with an MIC of 8 μg/ml), while the third *tet*(M)-positive isolate (HPA71) was initially sensitive to tetracycline (MIC of 1 μg/ml). However, subcultures from the lowest subinhibitory concentration of tetracycline found increased MICs for HPA111 (MIC of >64 μg/ml) and for Ply157 (MIC of 64 μg/ml), while HPA71 remained sensitive (MIC of 2 μg/ml). A second serial challenge with tetracycline found that resistance had been induced for HPA71 (MIC of 64 μg/ml). This induction of resistance in HPA71 was repeated twice with identical results. Therefore, screening for the presence of the *tet*(M) gene is less likely to miss resistant isolates than broth microdilution methods for tetracycline resistance. We sequenced the 3′ region of the *tet*(M) gene for the three isolates identified as *tet*(M) positive (two phenotypically resistant and one initially phenotypically sensitive). From this we determined that strains HPA71 and HPA111 were the most closely related to the previous Vancouver SV9 sequence, whereas strain Ply157 was a chimera of both the Vancouver and Seattle sequences (Table 4). No mutations within the 3′ region were identified to explain the required induction of tetracycline resistance for HPA71 (GenBank accession number KT267561). Susceptibility to doxycycline was similar to that observed for tetracycline for the resistant isolates (Table 2).

TABLE 3 Comparison of MIC_{50} and MIC_{90} values for various antibiotics between *U. parvum* and *U. urealyticum*

Antibiotic	Total no. of *Ureaplasma* drug-resistant samples	*U. parvum* (μg/ml)		*U. urealyticum* (μg/ml)		*P* value[a]
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	
Tetracycline	3	0.25	0.5	0.5	2	<0.001
Ciprofloxacin	2	1	2	2	4	<0.001
Erythromycin	0	1	2	2	4	<0.003
Azithromycin	0	0.25	0.25	0.25	0.25	NS
Chloramphenicol	0	2	4	2	4	NS
Gentamicin	130	32	64	64	128	<0.01

[a] The *P* value represents a Student *t* test comparison of the individual MIC values for all *U. parvum* isolates compared to the MIC values for all *U. urealyticum* isolates. NS, not significant.

Molecular mechanism for ciprofloxacin resistance. Molecular characterization was performed for two identified ciprofloxacin-resistant isolates using PCR sequencing of the QRDR as previously described (11, 13). The sequence analysis aligned to the published genome of *U. parvum* SV3 ATCC 700970 revealed two amino acid substitutions, V3D and E87K, in ParC of isolate U6 and a S83L ParC substitution in isolate HPA116.

Identification of resistance using the Mycoplasma IST2 test. The bioMérieux Mycoplasma IST2 kit was used to screen a subset of 20 submitted samples and the results for resistance to a spectrum of biologically active antibiotics. From the 20 samples examined, 8 were found to be *Ureaplasma* spp. positive, and all gave a reading of resistance to both the lower (1 μg/ml) and higher (2 μg/ml) levels for ciprofloxacin. The assay also showed that all *Ureaplasma* isolates were able to grow in 1 μg/ml of ofloxacin but not in the higher 4 μg/ml concentration. However, using the accepted international MIC broth microdilution technique, repeated in duplicate, three of these ciprofloxacin isolates had MICs of 1 μg/ml (identified as *U. parvum*), three had MICs of 2 μg/ml (identified as *U. parvum*), and two had MICs of 4 μg/ml (identified as *U. urealyticum*). The broth microdilution values determined that all of these isolates were sensitive to ciprofloxacin, consistent with the MIC_{90}s for their respective species (Table 3).

DISCUSSION

In recent years, *Ureaplasma* spp. have gained increasing recognition as pathogens in numerous clinical presentations. Due to the physiological properties of the organism and, in some cases, the patient populations, treatment options are highly restricted to only a few classes of antibiotics. Therefore, it is imperative that trends in resistance in both England and Wales and at an international level be monitored so that treatment options remain open. In this study, we report that antibiotic resistance to the three major classes of antibiotics used to treat *Ureaplasma* infections remains low in England and Wales.

TABLE 4 United Kingdom *tet*(M)-positive isolates compared with reference strains at the amino acid level

Isolate	Amino acid at position:						
	209	216	223	338	348	496	627
Vancouver	Q	L	S	K	T	D	Q
Seattle	H	V	N	R	I	E	R
HPA71	Q	L	S	K	T	D	Q
HPA111	Q	L	S	K	T	D	Q
Ply157C	H	V	N	K	T	D	Q

We last reported antibiotic resistance in *Ureaplasma* among isolates in England and Wales for samples collected before 2007 (11). At that time, 1.6% of isolates collected in England and Wales between 2003 and 2007 were resistant to one of the three main classes of antibiotics, and no dual resistance was identified. Here, from a larger cohort of 130 isolates, we report a similar level of resistance to ciprofloxacin (1.5%) and the presence of the tetracycline resistance gene (2.3%), whereas macrolide resistance was absent. This is a reassuringly low level of resistance compared with that in international reports. For example, Ye et al. reported 75% and 53% resistance to ciprofloxacin and ofloxacin, respectively (16). High levels of tetracycline resistance (73%) have been documented in South African studies as well as high levels of azithromycin resistance (29%) among patient cohorts in India (17, 18). This high level of macrolide resistance is of significant concern in the context of treating neonatal disease. Although comparisons can be made between studies, it is crucial to observe the methods used for detecting resistance. For example, Ye et al. used the Mycoplasma IST2 test, which from our data identified a number of false-positive results with regard to ciprofloxacin compared to the results for the standardized broth microdilution technique (16). In addition, the breakpoints and antibiotics used in this test are not in line with the recommended CLSI guidelines (5). In particular, the input inoculum for this assay is not standardized and cannot be measured by this assay, likely the cause of the false resistance results. Of interest from our data were the MIC values seen for *U. urealyticum*, which were significantly higher than those for *U. parvum* for most antibiotics tested. As *U. parvum* and *U. urealyticum* are recognized as two independent species, this is not a surprising finding.

Understanding the underlying mechanism of resistance is imperative. Sequence analysis of the QRDRs of isolate U6 identified two nonsynonymous mutations, resulting in the amino acid substitutions of V3D and E87K in the ParC protein. From our previous work cataloguing the species and serovar-specific differences, it is possible to definitively assign the E87K substitution to the phenotypic resistance (14). This substitution has been noted before in France by Bebear et al., who reported isolate UUc with the E87K substitution with a ciprofloxacin and ofloxacin MIC of 8 μg/ml (19). Interestingly although isolate U6 harbors the same point mutation as UUc, the MIC value was 4-fold greater. Previously, the V3D substitution may have been classified as contributing to the resistant phenotype of U6, yet this substitution appears to be a serovar-specific polymorphism whereby *U. parvum* SV3 and all serovars of *U. urealyticum* encode a valine residue, whereas serovars 1, 6, and 14 encode aspartic acid at position 3 for

ParC, although these data are based on a limited number of sequenced isolates (14). However, this observation has been further substantiated in our lab by examining whole-genome sequences for three additional SV3 strains, two SV6 strains, and one SV1 strain (our unpublished data). Irrespective of the serovar association (which may not hold as more sequences become available), the V3D substitution in ParC does not contribute to fluoroquinolone resistance as it exists in susceptible strains. The second ciprofloxacin-resistant strain (HPA116) was identified as harboring the predominant quinolone resistance-determining mutation S83L. This mutation has been described numerous times in patient cohorts from the United States, China, France, and Switzerland, but this is the first description among United Kingdom isolates (19–23). As the mechanism for quinolone resistance is mutation driven and not horizontally transferred, the likelihood of spread is limited as it would be clonal and could account for the relatively low level of resistance within these organisms.

Tetracycline resistance is well characterized among *Ureaplasma* and mediated via the acquisition of the *tet*(M) resistance element, giving ribosomal protection (24). As expected, all tetracycline-resistant strains in this study were positive for *tet*(M) in addition to a tetracycline-sensitive isolate (HPA71). By characterizing *tet*(M)-positive strains it is possible to track the emergence of new sequence variants within the United Kingdom. From these data, we identified two out of three *tet*(M)-positive strains to be identical to the Vancouver sequence, which we have previously described in the United Kingdom, but, curiously, the *tet*(M) sequence of isolate Ply157 was a chimera of both the Vancouver and Seattle strains. This is unlikely to be an artifact as it was confirmed by multiple sequencing experiments performed on this isolate. As with our study in 2009, we identified a single isolate which was *tet*(M) positive but phenotypically sensitive to the antibiotic (HPA71). We were successful in inducing expression and resultant resistance for this strain (but not other sensitive strains examined in parallel) with the presence of low levels of tetracycline in the culture medium. This brings into question the methods used for screening tetracycline resistance among *Ureaplasma* spp. When tetracycline resistance is examined, it may be necessary to screen by both culture and molecular methods to identify strains that harbor *tet*(M) variants, which require induction via the presence of the antibiotic. The inducible nature of some *tet*(M) genes has been previously reported in *Mycoplasma hominis*, but this is the first description among *Ureaplasma* spp. (25). From the three main classes of antibiotics active against *Ureaplasma* spp., tetracycline resistance poses a significant threat due to the horizontally transferable nature of the Tn*916*-like transposable element harboring the *tet*(M) gene and its potential to disseminate within a population.

We also compared the commercial Mycoplasma IST2 assay against the international broth microdilution methods as outlined by the Clinical and Laboratory Institute Standards (12). We found that the mixed isolation of *Ureaplasma* and *Mycoplasma hominis* of one sample showed as a false-positive macrolide resistance due to the intrinsic macrolide resistance seen among *M. hominis* and that all *Ureaplasma* spp.-positive samples were found to be resistant to the low (1 μg/ml) and high (2 μg/ml) concentrations of ciprofloxacin provided in the kit (26). However, broth microdilution evaluation found that three of the isolates had MICs of 1 μg/ml and 3 of the isolates had MICs of 2 μg/ml. All of these isolates were *U. parvum*. The remaining two isolates were *U. urealyticum* and had MICs of 4 μg/ml, which is in agreement with the slightly higher 95% CI determined to be between 2.64 and 3.66 μg/ml for ciprofloxacin. Therefore, none of the isolates were actually resistant to ciprofloxacin, relative to normal sensitivity ranges for the organisms tested, a result that questions the data obtained from this assay. Moreover, this could lead to inappropriate reporting of antibiotic resistance if the assay is used by researchers without a clear understanding of the internationally accepted methods and criteria for true antibiotic resistance.

Antibiotic resistance in England and Wales remains low. The high levels of resistance internationally pose a threat of importation into the United Kingdom and therefore continual surveillance is required to keep tract of resistance patterns. While it is tempting to attribute the continued low antibiotic resistance rates in the England and Wales to vigilance in keeping antibiotic prescription to a minimum, the geographic differential in antibiotic resistance is unlikely to be maintained, particularly with increasing travel between countries in combination with the increased prescribing of macrolide antibiotics for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Mycoplasma genitalium* infections.

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