Antibiotic resistance among clinical *Ureaplasma* isolates recovered from neonates in England and Wales between 2007 to 2013.

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Abstract:

Ureaplasma 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 sexual health clinic and 3 patients under investigation for immunological disorders, between 2007 – 2013 in England and Wales. Minimum inhibitory concentration was determined by using microbroth dilution assays, and a subset of isolates were compared using broth microdilution method and 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. MIC values for chloramphenicol were universally low (2 μg/mL), while inherently high level MIC values for gentamicin were seen (44-66 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.

Introduction:

Ureaplasma spp. are gaining recognition as a pathogen in both adult and neonatal patient groups. Availability of standardized molecular detection methods have increased the capacity to identify Ureaplasma in pathological conditions, which was previously difficult to identify by specialized culture-based methods. In adults *Ureaplasma* have been linked with non-gonococcal urethritis, arthritis, meningitis, chorioamnionitis and preterm labour whereas in neonates links have been made with bronchopulmonary dysplasia, neonatal pneumonia and meningitis (5, 6, 12, 17, 19, 21).

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 antibiotic which are recognized as active against ureaplasma are the quinolones, tetracyclines and macrolides. These treatment options are further limited in situations with neonates where the only recognized treatment is with a macrolide due to associated toxicity of the tetracyclines and quinolones (13).

Although two human associated *Ureaplasma* species have been recognized since 2002, *Ureaplasma urealyticum* and *Ureaplasma parvum*, many diagnostic laboratories still do not differentiate and report findings as *U. urealyticum* by default (16). This lack of discrimination hinders epidemiological data

and has partly been 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 non-gonococcal urethritis (NGU) whereas *U. parvum* does not (24). These data suggest that *U. urealyticum* may be a true pathogen in this situation whereas *U. parvum* represents a commensal.

In this report we describe the prevalence of antibiotic resistance among isolates of *Ureaplasma* 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* spp. isolates from anonymized unique patient samples originally submitted for clinical diagnostics between 2007 and 2013 were examined (Table 1). Species of *Ureaplasma* was determined by PCR as previously described (18). Sample source comprised a variety of patient groups: 61 neonatal endotracheal samples (15 *U. urealyticum* / 46 *U. parvum*) from 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 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 University Hospital of Wales (*U. parvum*).

Determination of antibiotic resistance with broth microdilution and Mycoplasma-IST2

Determination of minimum inhibitory concentration (MIC) and breakpoints were carried out as previously described by Beeton *et al.*,(4) which adheres to the Clinical and Laboratory Standards Institute guidelines (20). Antibiotics used for MIC were tetracycline, doxycycline, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, azithromycin, clarithromycin, chloramphenicol and gentamicin at a range of 0.06 µg/mL to 64 µg/mL. Breakpoint values were 2 µg/mL tetracycline, 4 µg/mL ciprofloxacin and 4 µg/mL azithromycin. Antibiotics were purchased from Sigma-Aldrich (Dorset, UK) and *Ureaplasma* selective media (USM) was supplied by Mycoplasma Experience Ltd (Surrey, UK). Twenty clinical samples submitted for testing to the Public Health England laboratory were examined in parallel to standard methods with the Mycoplasma-IST2 (bioMérieux, France) assay as per the manufacturer's instructions; eight were found to be positive for *Ureaplasma* spp. and identified resistance for ciprofloxacin for all isolates was followed up by appropriate broth microdilution methods.

PCR and sequencing of resistance genes

PCR and sequencing of the QRDR of ciprofloxacin resistant strains was carried out as previously described and aligned to the *U. parvum* SV3 reference genome of ATCC 700970 (2, 3). Confirmation of the *tet*(M) gene in tetracycline resistant strains was determined by PCR using the forward primer IntMtet1 located at position 309-328 bp and reverse primer tet2 located at position 832-851 bp in the coding region (Tm=55^{o}C, 35 cycles, amplicon = 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.(4, 8)

Statistical analysis

Statistics for the mean, standard deviation, standard error, and confidence intervals for MIC values for *U. parvum* and *U. urealyticum* were determined using GraphPad Prism and comparison of values between these species was performed via Students t-test.

Results:

Prevalence of resistance

Using the adapted broth microdilution technique we were able to identify two isolates resistant to ciprofloxacin (U6 32 μg/mL and HPA116 16 μg/mL), and three isolates which were tetracycline resistant (Table 1). This gave a prevalence of resistance of 1.5% (CI $\pm$ 2.09) and 2.3% (CI $\pm$ 2.58), respectively, for each antibiotic. No breakpoint values for resistance of ciprofloxacin are available in the CLSI guidelines (20); however, published breakpoints for moxifloxacin and levofloxacin indicate resistance to be $\geq$ 4 μg/mL. Both strains U6 and HPA116 were more sensitive to moxifloxacin (1 μg/mL) and levofloxacin (2 μg/mL) (Table 2), but these values were still higher than our susceptible strains: $\leq$0.25 μg/mL for moxifloxacin and $\leq$0.5 μg/mL for levofloxacin (data not shown). All 130 isolates were sensitive to the macrolide antibiotics erythromycin and azithromycin as well as chloramphenicol. All strains had an intrinsically high MIC for gentamicin (mean values of 42 μg/mL *U. parvum* and 66 μg/mLl *U. urealyticum*). No co-resistant strains were identified. The mean MIC of all antibiotics was significantly higher for *U. urealyticum* than *U. parvum* with exception of 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 isolates by PCR for the presence of the *tet*(M) gene and identified three positive strains of the 130 isolates (Table 4). Interestingly, broth culture screening for tetracycline resistance only identified two of these isolates (HPA111, MIC = 64 and Ply157, MIC = 8), while the third *tet*(M)-positive isolate was initially sensitive to tetracycline (HPA71 MIC = 1). However, subculture from the lowest sub-inhibitory concentration of tetracycline found increased MIC for HPA111 (MIC >64) and Ply157 (MIC=64), while HPA71 remained sensitive (MIC=2). A second serial challenge with tetracycline found that resistance had been induced for HPA71 (MIC=64). 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 microbroth dilution 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, one initially phenotypically sensitive). From this we identified that HPA71 and HPA111 were most closely related to the previous Vancouver SV9 sequence, whereas Ply157 was a chimera of both Vancouver and Seattle sequences (Table 4). No mutations within the 3' region were identified to explain the required induction of tetracycline resistance for HPA71 (accession number KT267561). Susceptibility to doxycycline was similar to that observed for tetracycline for the resistant isolates (Table 2).

Molecular mechanism for ciprofloxacin resistance

Molecular characterization was undertaken on two identified ciprofloxacin resistant isolates using previously described PCR-sequencing of the QRDR (2, 4). Sequence analysis aligned to the published genome of *U. parvum* SV3 ATCC 700970 revealed two amino acid substitutions of V3D and E87K in ParC of isolate U6 and a S83L ParC substitution in isolate HPA116.

Identification of resistance using the MIST2 test

The bioMérieux Mycoplasma IST2 kit was used to screen a subset of twenty 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* were able to grow in 1 µg/mL of ofloxacin, but not the higher 4 µg/mL concentration. However, using the accepted international MIC broth microdilution technique, repeated in duplicate, three of these ciprofloxacin isolates had an MIC = 1 µg/mL (identified as *U. parvum*), three had an MIC = 2 µg/mL (identified as *U. parvum*) and two had an MIC = 4 µg/mL (identified as *U. urealyticum*). The microbroth dilution values determined that all of these isolates were sensitive to ciprofloxacin and consistent with the MIC_{90} for their respective species (Table 3).

Discussion

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

We last reported the prevalence of antibiotic resistance in *Ureaplasma* among isolates in England and Wales for samples collected before 2007 (4). At this point in 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 presence of the tetracycline resistance gene (2.3%), whereas macrolide resistance was absent. This is a reassuringly low level of resistance when compared with international reports. For example, Ye *et al*. reported 75% and 53% resistance to ciprofloxacin and ofloxacin, respectively (23). 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 (10, 15). 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 to detecting resistance. For example Ye *et al.*, used the Mycoplasma IST2 test, which from our data identified a number of false positive results with regards to ciprofloxacin when compared to the standardized microbroth dilution technique (23). In addition the breakpoints and antibiotics used in this test are not in line with the recommended CLSI guidelines (20). 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 was the MIC values seen for *U. urealyticum* were significantly higher when compared with *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 non-synonymous mutations resulting in the amino acid substitutions of V3D and E87K in 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 (3). 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 (1). 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 is now known as 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 three for ParC. It is unlikely that the E87K substitution requires this second V3D substitution to exert the phenotypic resistance due to the absence of the aspartic acid residue in the French UUc strain. The second ciprofloxacin resistant strain (HPA116) was identified to harbor the predominant quinolone resistance determining mutation S83L. This mutation has been described numerous times from patient cohorts from the USA, China and France, but this is the first description among UK isolates (1, 11, 22, 25). 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 (7). 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 UK. 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 UK, but curiously the *tet*(M) sequence of isolate Ply157 was a chimera of both Vancouver and Seattle strains. This is unlikely 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*. When examining tetracycline resistance it may be necessary to screen by both culture and molecular methods to identify strains which harbor *tet*(M) variants which require induction via 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* (9). From the three main classes of antibiotics active against *Ureaplasma*, tetracycline

poses a significant threat due to the horizontally transferable nature of the mobile element and its potential to disseminate within a population.

We also compared the commercial assay Mycoplasma-IST2 against the international broth microdilution methods as outlined by the Clinical and Laboratory Institute Standards (20). We found that 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* 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 (14). However, broth microdilution evaluation of these found that three of the isolates had an MIC = 1 µg/ml and 3 of the isolates had an MIC = 2 µg/mL. All of these isolates were *U. parvum*. The remaining two isolates were *U. urealyticum* and had an MIC = 4 µg/mL which is within keeping with the slightly higher CI_{95} determined to be between 2.64-3.66 µg/mL for ciprofloxacin. Therefore, none of the isolates were actually resistant to ciprofloxacin, relative to normal sensitivity ranges for the organisms tested and questions the data obtained from this assay. Moreover, it could lead to inappropriate reporting of antibiotic resistance if 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 poses a threat of import into the UK 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 *N. gonorrhoeae, Chlamydia trachomatis* and *Mycoplasma genitalium* infections. An international

collaborative working group to ascertain and provide known susceptible and resistant control strains for validation to laboratories for use in clinical assays and research prior to publication of results, along with increased reviewer scrutiny could increase antibiotic resistance determination following internationally accepted MIC methods and ensure the molecular identification of proven mechanisms of resistance and importantly the essential differentiation of *Ureaplasma* species.

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Transparency declarations

None to declare

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366 Table 1. Source and year of isolation for *Ureaplasma* species used for MIC determination.

Source	2007	2008	2009	2010	2011	2012	2013	total
PHE (PCR +)	N/A	N/A	N/A	28	47	33	60	168
PHE (PCR -)	N/A	N/A	N/A	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

367 Legend: Samples were obtained from Public Health England (PHE), University Hospital of Wales
368 neonatal intensive care unit (UHW) or immunological out-patients (Urine), Derriford Hospital
369 neonatal intensive care unit (Plymouth) or the Royal Glamorgan Hospital (RGH). Not all PHE isolates
370 were recoverable from frozen archives for MIC determination. N/A = data not available.

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Table 2. Overview of antibiotic resistant isolates identified from UK samples between 2007 to 2013.

Isolate	Species of *Ureaplasma*	Antibiotic resistance (MICµg/mL)	Mechanism of resistance
U6	*U. parvum*	Ciprofloxacin (32) Levofloxacin (2) Moxifloxacin (1)	E87K in ParC
HPA116	*U. parvum*	Ciprofloxacin (16) Levofloxacin (2) Moxifloxacin (1)	S83L in ParC
HPA111	*U. urealyticum*	Tetracycline (64) Doxycycline (16)	*Tet*(M) positive
PLY157	*U. parvum*	Tetracycline (8) Doxycycline (8)	*Tet*(M) positive
HPA71	*U. urealyticum*	Tetracycline (64*) Doxycycline (16*)	*Tet*(M) positive

* MIC following challenge with tetracycline (initial MIC = 1µg/mL)

381 Table 3. Comparison of MIC_{50} and MIC_{90} concentrations of various antibiotics between *U. parvum*
382 and *U. urealyticum*.
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Antibiotic	Total *Ureaplasma* resistant	*U. parvum*		*U. urealyticum*		p-value*
		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

384 P-value represents a student's t-test comparison of the individual MIC values for all *U. parvum*
385 isolates compared to the MIC values for all *U. urealyticum* isolates.
386
387 Table 4. UK *tet*(M) positive isolates compared with reference strains at the amino acid level

Isolate	Amino acid 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

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