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Fluoroquinolone and Macrolide Resistance-Associated Mutations in *Mycoplasma genitalium*

Kaitlin A. Tagg,[a,b] Neisha J. Jeoffreys,[b] Deborah L. Couldwell,[c,d] Jennifer A. Donald,[a] Gwendolyn L. Gilbert[b,d]

Department of Biological Sciences, Macquarie University, North Ryde, NSW, Australia[a]; Centre for Infectious Diseases and Microbiology, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, NSW, Australia[b]; Western Sydney Sexual Health Centre, Westmead Hospital, NSW, Australia[c]; Sydney Emerging Infections and Biosecurity Institute, University of Sydney, Sydney, NSW, Australia[d]

Mycoplasma genitalium **is a significant sexually transmitted pathogen, causing up to 25% of cases of nongonococcal urethritis in men, and it is strongly associated with cervicitis and pelvic inflammatory disease in women. Currently, the usual first-line treatment is the macrolide antibiotic azithromycin, but an increasing incidence of treatment failure over the last 5 years suggests the emergence of antibiotic resistance. The mutations responsible for macrolide resistance have been found in the 23S rRNA gene in numerous *M. genitalium* populations. A second-line antibiotic, the fluoroquinolone moxifloxacin, was thought to be a reliable alternative when azithromycin began to fail, but recent studies have identified mutations that may confer fluoroquinolone resistance in the genes *parC* and *gyrA*. The aim of this study was to determine the prevalence of antibiotic resistance in *M. genitalium* in Sydney, Australia, by detecting relevant mutations in the 23S rRNA gene, *parC*, and *gyrA*. *M. genitalium*-positive DNA extracts of specimens, collected from patients attending sexual health clinics in Sydney, were tested by PCR amplification and DNA sequence alignment. The 186 specimens tested included 143 initial patient specimens and 43 second, or subsequent, specimens from 24 patients. We identified known macrolide resistance-associated mutations in the 23S rRNA gene in 43% of the initial patient samples and mutations potentially associated with fluoroquinolone resistance in *parC* or *gyrA* sequences in 15% of the initial patient samples. These findings support anecdotal clinical reports of azithromycin and moxifloxacin treatment failures in Sydney. Our results indicate that further surveillance is needed, and testing and treatment protocols for *M. genitalium* infections may need to be reviewed.**

Mycoplasma genitalium, one of the smallest known self-replicating organisms (1), is an obligate parasite, preferring the human genital tract as its host environment (2–4). In men, *M. genitalium* is a significant cause of both acute and chronic nongonococcal urethritis (NGU) (5–7) and is estimated to account for 15% to 25% of cases of NGU in some populations (2). In women, *M. genitalium* has been significantly associated with both cervicitis (2, 8–10) and pelvic inflammatory disease (2, 11–14), and it has been identified in up to 7.3% of women in high-risk populations (15). In Australia, *M. genitalium* has been reported to account for between 4.5% and 9% of cases of NGU (16, 17), while it was identified in 4% of women attending a Sydney sexual health clinic and was the second most common cause of cervicitis (9).

The usual treatment regime for clinically diagnosed NGU and cervicitis is a single dose of 1 g azithromycin (AZM) (18). An extended regime (1.5 g over 5 days) is often prescribed if single-dose AZM is unsuccessful, but there is contention regarding the effectiveness of this extended treatment (19, 20). Alarmingly, studies investigating the effectiveness of either 1 g or 1.5 g AZM treatment have reported microbiological treatment failure in 16% to 33% of patients (19, 21–24). Point mutations at positions 2058 and 2059 (*Escherichia coli* numbering) in region V of the 23S rRNA gene (25, 26) are consistently identified in *M. genitalium* strains taken from patients in whom AZM treatment has failed; these strains exhibit high levels of AZM resistance *in vitro* (25, 27–29).

Moxifloxacin (MXF) is used as a second-line treatment for *M. genitalium*-associated NGU and cervicitis (21, 30) and is consistently found to be among the most active drugs against *M. genitalium in vitro* (31, 32). Clinical resistance to MXF has not yet been formally reported in *M. genitalium* infections. However, a recent study has identified point mutations in the quinolone resistance-determining region (QRDR) (33) of the topoisomerase IV gene *parC* in 11% of *M. genitalium* clinical specimens, suggesting the emergence of fluoroquinolone resistance (34). In addition, mutations in the QRDR of the DNA gyrase gene, *gyrA*, have been identified in fluoroquinolone-resistant strains of *Mycoplasma pneumoniae*, the closest relative to *M. genitalium* (35).

Little is known about the incidence of AZM and MXF resistance in *M. genitalium* in Sydney, Australia, despite the regular use of these antibiotics. The aim of this study was therefore to estimate the prevalence of resistance to these antibiotics in *M. genitalium*-positive specimens taken from patients attending sexual health clinics. PCR amplification and DNA sequencing were utilized to detect the presence of potential resistance-inducing mutations in the 23S rRNA gene, *parC*, and *gyrA*.

MATERIALS AND METHODS

This retrospective study was approved by the Human Research Ethics Committee, Western Sydney Local Health District (Westmead, Australia). All clinical specimens were collected from patients attending sexual health clinics in Sydney, Australia, between 2008 and 2011. All processing and testing of samples for the detection of *M. genitalium* were performed at the Centre for Infectious Diseases and Microbiology (CIDM) Labora-

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Address correspondence to Kaitlin A. Tagg, ktag4715@uni.sydney.edu.au.

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TABLE 1 Optimum PCR concentrations and conditions

Parameter	Value			
Master mix PCR reagent	23S rRNA	*parC*	*gyrA*	*gyrA*2
HotStarTaq master mix	1×	1×	1×	1×
Forward primer (nM)	200	200	200	150
Reverse primer (nM)	200	200	200	150
$MgCl_2$ (mM)	1	2	2	0
DNA (10 μl)	1:10	1:100	1:10	1:10
Total vol (μl)	25	25	25	25
PCR step	Temp (°C)	Time	No. of cycles	
Activation	95	10 min	1	
Denaturation	95	10 s	40	
Annealing	—[a]	30 s	40	
Extension	72	30 s	40	
Final	72	5 min	1	

[a] Annealing temperatures were 65°C for the 23S rRNA gene, 64°C for *parC*, 62°C for *gyrA*, and 61°C for *gyrA*2.

tory Services, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital (Westmead, Australia). Samples were analyzed without knowledge of the identity of the patients or the clinic from which the samples were collected.

DNA extraction, amplification, and sequencing. DNA was extracted from 186 clinical specimens (urine or urethral or cervical swabs) using the NucliSENS easyMag (bioMérieux, France), according to the manufacturer's instructions. Extracts were then tested for the presence of *M. genitalium* DNA using an in-house PCR assay targeting *mgpB*, and those that were positive were frozen at −20°C with an aliquot of the original specimen.

The primers used for the amplification of the 23S rRNA gene (25), *parC*, and *gyrA* (34) have been described previously. In addition, a second *gyrA* primer set that did not contain degenerate nucleotides, gyrktF (5′-GCTCGTGCTTTACCTGATGCTAGA-3′) and gyrktR (5′-AACGTTGTGCAGCAGGTCT-3′), was designed to increase the sensitivity of the *gyrA* assay. The PCR reagents, their concentrations, and the conditions for each assay are shown in Table 1. The specificities of each assay were evaluated using a panel of urogenital, commensal, and closely related organisms, namely, *Candida albicans*, *Chlamydia trachomatis*, *Gardnerella vaginalis*, *Mycoplasma hominis*, *M. pneumoniae*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Treponema pallidum*, and *Ureaplasma urealyticum*. PCR products were separated by agarose gel electrophoresis.

Amplified products were cleaned using illustra ExoStar 1-step PCR clean up solution (GE Healthcare, United Kingdom) and were sequenced at the Australian Genome Research Facility (AGRF), Westmead Millennium Institute (Westmead, Australia), on the Applied Biosystems 96-capillary 3730xl DNA analyzer (Life Technologies). FASTA sequences were aligned against the five *M. genitalium* complete genome sequences available in GenBank, i.e., for strains G37, M2288, M2321, M6282, and M6320 (GenBank accession no. NC_000908.2, CP003773.1, CP003770.1, CP003771.1, and CP003772.1, respectively; http://www.ncbi.nlm.nih.gov), and were analyzed using NCBI BLAST and UniPro UGENE 1.11 bioinformatics software (UniPro, Russia).

Patient information for clinical samples. Samples were identified by unique patient-specific numbers, and the date and site of specimen collection were recorded for each. The laboratory database was searched to identify first and subsequent *M. genitalium*-positive specimens from individual patients. Only first-test-positive samples were used to estimate the prevalence of resistance, defined by the proportion of *M. genitalium*-infected patients who harbored resistant strains in their first samples.

Statistical tests were performed using chi-square or Fisher's exact tests to analyze the differences in the resistance-associated mutation rates between samples from different time periods (GraphPad Software, Inc.).

RESULTS

Alignment of the five *M. genitalium* complete genomes showed that, compared with G37, one strain (M6320) harbored an A2059G mutation in the 23S rRNA gene and is therefore likely to be resistant to azithromycin, and another strain (M6282) harbored both a silent mutation (C234T) and a missense mutation (C184T) in the QRDR of *parC*. The C234T mutation was also found commonly in our specimens (Table 2), but the significance of the C184T missense mutation is unknown. There were no other sequence differences between the five strains within the three amplified regions of interest, suggesting that the differences identified in our study specimens are significant.

Mutations identified in this study, and their resulting amino acid changes, are shown in Table 2. Four different mutations were identified in the 23S rRNA gene sequences at position 2058 or 2059 (*E. coli* numbering). Nine different missense mutations were identified in the QRDR of *parC* and one was identified in the QRDR of *gyrA*.

DNA extracts of *C. albicans*, *C. trachomatis*, *G. vaginalis*, *M. hominis*, *M. pneumoniae*, *N. gonorrhoeae*, *T. vaginalis*, *T. pallidum*, and *U. urealyticum* did not produce visible amplicons of any size in the 23S rRNA gene, *parC*, or *gyrA* assays.

Correlation of results and patient information. The 186 positive samples tested in this study were from 149 patients, from whom only the initial samples were used to calculate mutation frequencies. Of these, 143 patients had results available for all three genes and were further analyzed. The gene mutation profiles of these 143 patients are shown in Table 3. Sixty-two patients (43.4%) harbored *M. genitalium* strains with 23S rRNA gene mutations, while 22 (15.4%) had *parC* (21 patients) or *gyrA* (one patient) mutations that resulted in amino acid changes at positions that have been previously associated with fluoroquinolone resistance (Table 2).

Between 2008 and 2010, *M. genitalium* PCR was performed only by physician request after multiple NGU treatment failures. From 2011, it was performed routinely on all specimens from patients with NGU. Therefore, we compared the mutation rates for the first specimens collected in these two periods separately, with the following results: for the 23S rRNA gene in 2008 to 2010, 35/68 (51.5%), compared to the rate in 2011, 27/75 (36.0%) ($P = 0.06$); for *parC* and *gyrA* (combined) in 2008 to 2010, 12/68 (17.6%), compared to the rate in 2011, 10/75 (13.3%) ($P = 0.5$).

Twenty-four patients had a second or subsequent PCR-positive sample available for testing, of whom 23 (95.8%) harbored a 23S rRNA gene mutant, including eight (34.8%) who had wild-type profiles in their first samples. One sample repeatedly showed an indeterminate A/G nucleotide at position 2058, potentially representing a mixed population of mutant and wild-type strains (30), but it was not analyzed further. A second PCR-positive sample was available for three (13.6%) of the 22 patients with potential fluoroquinolone resistance mutations, and all harbored the same mutation profile as the first.

DISCUSSION

Moxifloxacin resistance: *parC* and *gyrA* mutations. For the first time in Sydney, Australia, the prevalence of *M. genitalium* strains that are potentially resistant to fluoroquinolones was estimated. By combining the number of mutations in *parC* and *gyrA* that have been previously linked to fluoroquinolone resistance, we estimated the prevalence of potentially resistant strains in Sydney

TABLE 2 Position and frequency of point mutations identified in each gene of interest

Gene	Position[a]	Mutation	Amino acid change	No. of first samples[b] (n = 143 for each gene)	Frequency (%)[b,c]	Reference no.[d]
23S rRNA						
	2071 (2058)	A→C		1	0.7	25
		A→G		21	14.7	25
		A→T		2	1.4	30
	2072 (2059)	A→G		38	26.6	25
Total				62/143	43.4	
parC						
	234	C→T*	No change	27*	18.9*	
	241	G→T	Gly→Cys 81(78)	1	0.7	35
	244	G→A	Asp→Asn 82(79)	1	0.7	36
	247	A→C	Ser→Arg 83(80)	1	0.7	34
	248	G→T	Ser→Ile 83(80)	11	7.7	35
	259	G→A	Asp→Asn 87(84)	3	2.1	35
		G→C	Asp→His 87(84)	1	0.7	34
		G→T	Asp→Tyr 87(84)	2	1.4	34
	260	A→G	Asp→Gly 87(84)	1	0.7	35
	307	G→A[e]	Val→Ile 103(100)[e]	1	0.7	
	351	T→C*	No change	1*	0.7*	
Total				22/143	15.4	
gyrA						
	237	G→T*	No change	1*	0.7*	
	270	C→T*	No change	1*	0.7*	
	285	G→A*	No change	4*	2.8*	
		G→C	Met→Ile 95(83)	1	0.7	35, 37
Total				1/143	0.7	

[a] Position numbers are given according to the *M. genitalium* G37 genome (GenBank accession no. NC_000908.2). *E. coli* numbering is shown in parentheses.
[b] Asterisks indicate mutations that were not included in the total due to lack of an amino acid change.
[c] Frequencies are total number of mutations, irrespective of whether a mutation is associated with antibiotic resistance.
[d] Reference for identification of the mutation associated with resistance at the same position in *M. genitalium* or a closely related organism.
[e] Amino acid position not previously associated with antibiotic resistance.

sexual health clinics to be 15%. In 2011, when specimens were tested routinely, the rate was 13%; although lower, this result was not significantly different from that of the earlier period. This rate is similar to a recent estimate in Japan of 10% (n = 28) in clinical populations (34).

The *parC* missense mutations included in this estimate occurred at amino acid positions 78, 79, 80, and 84, which are all positions that are known to be associated with fluoroquinolone resistance in *M. genitalium* and other closely related organisms (34–37). We did not perform susceptibility testing to confirm phenotypic resistance. However, based on previous studies, it is likely that these changes contribute to MXF resistance (34, 35).

TABLE 3 Mutation profiles identified in 143 first-test patient samples

Mutation profile	Gene mutation			No. of patients
	23S rRNA	*parC*	*gyrA*	
Wild type	−	−	−	73
AZM mutation	+	−	−	48
MXF mutation	−	+	−	8
AZM+MXF mutation	+	+	−	13
AZM+MXF mutation	+	−	+	1

The amino acid changes Gly-78→Cys and Asp-79→Asn were demonstrated here for the first time in *M. genitalium*. They have been previously linked to fluoroquinolone resistance in *M. pneumoniae* and *U. urealyticum*, respectively (35, 36). To the best of our knowledge, the Val-100→Ile mutation identified in this study has not been linked to resistance in any closely related organisms. The only mutation identified in the QRDR of *gyrA*, Met-83→Ile, also has not been reported previously in *M. genitalium*. However, mutations at position 83, which is a "hot spot" for fluoroquinolone resistance (38), have been reported in MXF-resistant strains of *M. pneumoniae*, *M. hominis*, and *Ureaplasma* spp. (35, 37).

Without subtyping of these strains, it was not possible to determine whether the *parC* and *gyrA* mutants were the result of *de novo* mutation events that were then selected by the use of fluoroquinolones or were descendants of clonal mutant lineages. Nonetheless, the presence of these mutations in clinical strains of *M. genitalium* raises concerns about the potential for fluoroquinolone resistance to emerge in the community.

Azithromycin resistance: 23S rRNA gene mutations. Identification of mutations in the 23S rRNA gene at nucleotides 2058 and 2059, positions known to be linked to high-level AZM resistance (25, 26), allowed for the first estimate of the prevalence of AZM resistance in *M. genitalium* in Sydney, Australia. The overall prevalence was 43%, with a lower prevalence in 2011 (36%) than

in 2008 to 2010 (51%) (although the difference is not statistically different). Another recent estimate of *M. genitalium* resistance from Melbourne, Australia, was 19.5% ($n = 82$) (30), which is much lower than that found in this study, although both estimates demonstrate alarmingly high levels of *M. genitalium* AZM resistance in patients attending sexual health clinics.

High levels of resistance may be the result of frequent selection of resistant strains by a 1-g AZM dose (30), as several studies have shown that selection of resistant strains commonly occurs over the course of 1-g AZM treatment (25, 27, 29). Alarmingly, a very recent study reported that selection over the course of treatment accounted for 55% of AZM treatment failure cases (30). This is concerning, as mycoplasmas have a high mutation rate that is likely to rapidly generate mutants (3), thus allowing selection to occur at an accelerated rate (30). The continuing use of 1 g AZM to treat *M. genitalium* infections will increase the incidence of resistance (30), leaving us increasingly more reliant on MXF; this is a concerning fact, given that this study suggests the emergence of fluoroquinolone resistance in Sydney.

As this is a retrospective study, there are both clinical and molecular limitations to consider. The estimated frequency of resistance was based on the assumption that patient first-test samples were collected before exposure to AZM or MXF; thus, the mutation rates are probably overestimated. We have attempted to reduce this overestimation by using only 2011 samples, since the 2011 testing protocol allowed sexual health clinics to test for *M. genitalium* upon first presentation of NGU or cervicitis, rather than after treatment failure. However, patients who were tested in 2011 may still have visited a general practitioner for treatment before presenting to a sexual health clinic for testing.

Molecular limitations are those that are commonly associated with retrospective studies, including DNA degradation in stored samples and the lack of original specimens for reextraction. DNA degradation could have been minimized by storing samples at −80°C (39). For future amplification of *gyrA*, we would recommend the more-sensitive *gyrA2* assay, described for the first time in this study.

In future studies, cultures should be attempted with antibiotic susceptibility testing and subtyping of isolates to determine the resistance phenotypes of *parC* and *gyrA* mutants and whether they are *de novo* mutants or descendants of clonal mutant lineages. This would enable a better understanding of the most clinically relevant mutations. Furthermore, we did not test for other mechanisms of resistance, such as mobile genetic elements, but to the best of our knowledge, alternate resistance mechanisms have not been reported in *M. genitalium* (3).

In conclusion, this study provides the first estimate of potential MXF resistance in Sydney, Australia, to be 13% and identifies AZM resistance mutations in 36% of clinical samples of *M. genitalium*. How these mutant strains began to emerge in Sydney cannot be determined, although we can employ the knowledge gained from this study as the basis for review of NGU and cervicitis treatment protocols, to promote antibiotic stewardship, to monitor and limit the spread of resistance, and to reduce patient morbidity.

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