Fluoroquinolone and macrolide resistance-associated mutations in *Mycoplasma genitalium*

Antibiotic resistance in *Mycoplasma genitalium*

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ABSTRACT

*Mycoplasma genitalium* is a significant sexually transmitted pathogen, causing up to 25% of cases of non-gonococcal urethritis in men and is strongly associated with cervicitis and pelvic inflammatory disease in women. Currently, the usual first-line treatment is with the macrolide antibiotic, azithromycin, but an increasing incidence of treatment failure over the last five years suggests emergence of resistance. Mutations responsible for macrolide resistance have been found in the 23S rRNA gene (23S rDNA) in numerous *M. genitalium* populations. A second-line antibiotic, the fluoroquinolone moxifloxacin, was thought to be a reliable alternative when azithromycin failed, but recent studies have identified mutations in the genes, *parC* and *gyrA*, that may confer fluoroquinolone resistance.

The aim of this study was to determine the prevalence of antibiotic resistance in *M. genitalium* in Sydney, Australia, by detection of relevant mutations in 23S rDNA, *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 23S rDNA of 43%, and mutations potentially associated with fluoroquinolone resistance in *parC* or *gyrA* sequences of 15%, of initial patient samples. These findings support anecdotal clinical reports of azithromycin and moxifloxacin treatment failures in Sydney. Our results indicated that
further surveillance is needed and testing and treatment protocols for *M. genitalium* infections may need to be reviewed.

**INTRODUCTION**

*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 non-gonococcal 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 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 azithromycin (AZM) 1 g single dose (18). An extended regime (1.5 g over five 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 rDNA (25, 26) are consistently identified in *M. genitalium* strains taken from patients in whom AZM
treatment has failed, and these strains exhibit high level 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 infection. 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 their regular use. 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 utilised to detect the presence of potential resistance-inducing mutations in 23S rDNA, 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 detection of *M. genitalium* was performed at the Centre for Infectious Diseases and Microbiology (CIDM) Laboratory Services, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital (Westmead, Australia). Samples were analysed without knowledge of the identity of the patient, or the clinic from which the samples were collected.

**DNA extraction, amplification and sequencing**

DNA was extracted from 186 clinical specimens (urine, urethral or cervical swabs) using the NucliSENS® easyMAC® (bioMérieux, France), according to the manufacturer’s instructions. Clinical specimens had been tested for the presence of *M. genitalium* DNA, on receipt, 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 amplification of 23S rDNA (25), *parC* and *gyrA* (34) have been previously described. 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*, *Mycoplasma 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, UK) 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™, USA). FASTA sequences were aligned against the five *M. genitalium* complete genome sequences available on GenBank - G37, M2288, M2321, M6282 and M6320, (GenBank accession numbers NC_000908.2, CP003773.1, CP003770.1, CP003771.1, CP003772.1: http://www.ncbi.nlm.nih.gov) and were analysed 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. 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 harboured resistant strains in their first samples.

Statistical tests were performed using chi square or Fisher’s exact test, to analyse the differences in resistance-associated mutation rates between different time periods (GraphPad Software, Inc., USA).
RESULTS

Alignment of the five *M. genitalium* complete genomes showed that, when compared with G37, one strain (M6320) harboured a A2059G mutation in the 23S rDNA and is therefore likely to be resistant to azithromycin, and another strain (M6282) harboured 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 23S rDNA at two positions - 2058 or 2059 (*E. coli* numbering). Nine different missense mutations were identified in the QRDR of *parC* and one 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 rDNA, *parC* or *gyrA* assays.

Correlation of results to patient information

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

Between 2008 and 2010, *M. genitalium* PCR was performed only on physician request after multiple NGU treatment failures. From 2011, it was performed routinely on all specimens from patients with NGU. Therefore, we compared mutation rates for first specimens collected in these two periods, separately, with the following results: 23S rDNA: 2008-2010, 35/68 (51.5%), compared to 2011, 27/75 (36.0%) (p=0.06); *parC* and *gyrA* (combined), 2008-10, 12/68 (17.6%), compared to 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%) harboured a 23S rDNA 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 was not further analysed. A second PCR-positive sample was available for three (13.6%) of the 22 patients with potential fluoroquinolone resistance mutations and all harboured the same mutation profile as the first.
DISCUSSION

**Moxifloxacin resistance: parC and gyrA mutations**

For the first time in Sydney, Australia, the frequency of *M. genitalium* strains potentially resistant to fluoroquinolones was estimated. By combining the number of mutations previously linked to fluoroquinolone resistance in *parC* and *gyrA*, we estimated the prevalence of potentially resistant strains in Sydney sexual health clinics to be 15%. In 2011, when specimens were tested routinely, the rate was 13%, but although less, this result was not significantly different from that during the earlier period. This rate is similar to a recent estimate in Japan of 10% (n=28) in clinical populations (34).

The *parC* amino acid changes included in this estimate occurred at positions 78, 79, 80, and 84, which are all positions known to be associated with fluoroquinolone resistance in *M. genitalium* and other closely related organisms (34, 35, 37, 38). 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).

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, 37). 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 in *M. genitalium* previously. However, mutations at position 83, a “hot spot” for fluoroquinolone resistance (39), have been
reported in MXF-resistant strains of *M. pneumoniae*, *M. hominis* and *Ureaplasma* spp. (35, 38).

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 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 23S rDNA at nucleotides 2058 and 2059, positions known to be linked to high level AZM resistance (25, 26), allowed 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 2008-2010 (51%) (difference not statistically different). Another recent estimate of *M. genitalium* resistance from Melbourne, Australia, was 19.5% (n=82) (30), much lower than 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 AZM 1 g dose (30), as several studies have shown that selection of resistant strains commonly occurs over the course of AZM 1 g 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 generate mutants rapidly (3), thus allowing selection to occur at an accelerated rate (30). The continuing use of AZM 1 g to treat *M. genitalium* infections will increase the incidence of resistance (30), leaving us increasingly more reliant on MXF - a concerning fact, given that this study suggests the emergence of fluoroquinolone resistance in Sydney.

As this was a retrospective study, there were 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 in 2011 may still have visited a general practitioner for treatment before presenting to a sexual health clinic for testing.

Molecular limitations were those commonly associated with retrospective studies, including DNA degradation of stored samples and lack of original specimens for re-extraction. DNA degradation could have been minimised by storing samples at -80°C (40). For future amplification of *gyrA* we would recommend the more sensitive *gyrA*2 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 provided the first estimate of potential MXF resistance in Sydney, Australia, at 13%, and identified 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.

**ACKNOWLEDGEMENTS**

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We would like to acknowledge Yasushi Shimada and Takashi Deguchi, who both so kindly responded to queries regarding their published papers.

We certify that there are no conflicts of interest in relation to this work.
REFERENCES


### Table 1. Optimum PCR concentrations and conditions

Master mix PCR reagents and concentrations for each assay

<table>
<thead>
<tr>
<th>Reagent (stock concn)</th>
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<th>parC</th>
<th>gyrA</th>
<th>gyrA2</th>
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<td>HotStarTaq® Master Mix (2X)</td>
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<td>1X</td>
<td>1X</td>
<td>1X</td>
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<td>200 nM</td>
<td>200 nM</td>
<td>150 nM</td>
</tr>
<tr>
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<td>200 nM</td>
<td>200 nM</td>
<td>150 nM</td>
</tr>
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</tr>
<tr>
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<td>1:100</td>
<td>1:10</td>
<td>1:10</td>
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<tr>
<td>Total vol (µL)</td>
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**PCR conditions**

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</tr>
<tr>
<td>Denaturation</td>
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<td>Annealing</td>
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**NOTE** * Annealing temperatures were 65°C - 23S rDNA; 64°C - parC; 62°C - gyrA; 61°C - gyrA2.
<table>
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<th>Gene</th>
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<th>Amino acid change</th>
<th>No. of first samples</th>
<th>Frequency (%)</th>
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<td>23S rDNA</td>
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<td>2071(2058)</td>
<td>A→C</td>
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<td>1</td>
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<td>(25)</td>
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<tr>
<td></td>
<td>A→G</td>
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<td></td>
<td>21</td>
<td>14.7</td>
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<td>A→T</td>
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<td>1.4</td>
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</tr>
<tr>
<td>2072(2059)</td>
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<td></td>
<td>38</td>
<td>26.6</td>
<td>(25)</td>
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<tr>
<td>Total</td>
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<td></td>
<td></td>
<td>62/143</td>
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<tr>
<td>parC</td>
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<td>(34)</td>
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<td>(35)</td>
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<td>Val→Ile 103(100γ)</td>
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<td>gyrA</td>
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<td>1*</td>
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<td></td>
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<tr>
<td>285</td>
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<td>4*</td>
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<td></td>
<td>G→C</td>
<td>Met→Ile 95(83)</td>
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<td>0.7</td>
<td>(35, 38)</td>
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<td>Total</td>
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</table>

**NOTE** Position numbers are given according to *M. genitalium* G37 genome, GenBank accession number NC_000908.2.

* E. coli numbering is shown in parentheses.

Frequencies are total no. of mutations, irrespective of whether mutation is associated with antibiotic resistance.

Reference for identification of mutation associated with resistance at same position in *M. genitalium* or closely related organism.

* Mutations not included in total due to no amino acid change.

γ Amino acid position not previously associated with antibiotic resistance.
<table>
<thead>
<tr>
<th>Mutation profile</th>
<th>23S rDNA</th>
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<th>gyrA</th>
<th>No. of patients</th>
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<tr>
<td>AZM mutation</td>
<td>+</td>
<td>...</td>
<td>...</td>
<td>48</td>
</tr>
<tr>
<td>MXF mutation</td>
<td>...</td>
<td>+</td>
<td>...</td>
<td>8</td>
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<td>AZM+MXF mutation</td>
<td>+</td>
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<td>...</td>
<td>13</td>
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Table 3. Mutation profiles identified in 143 first-test patient samples