Azithromycin Treatment Failure in *Mycoplasma genitalium*–Positive Patients with Nongonococcal Urethritis Is Associated with Induced Macrolide Resistance

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(See the editorial commentary by Taylor-Robinson on pages 1554–5)

**Background.** *Mycoplasma genitalium* is a common cause of nongonococcal urethritis. Treatment trials have shown that doxycycline is inefficient, whereas a 5-day course of azithromycin eradicates the bacterium from 95% of infected men. The aim of the study was to establish the reason for the occasional treatment failures.

**Methods.** Seven *M. genitalium* strains isolated from men who experienced azithromycin treatment failure were tested for in vitro susceptibility to macrolides with use of a cell culture–based method. The genetic basis for the drug resistance was established by sequencing parts of the 23S ribosomal RNA gene and the genes encoding the L4 and L22 proteins. Nine sets of specimens obtained before and after treatment from patients who experienced azithromycin treatment failure were examined with use of sequencing of polymerase chain reaction products.

**Results.** The 7 strains that were isolated from patients who experienced treatment failure with azithromycin had minimum inhibitory concentrations ≥8 μg/mL for azithromycin and erythromycin. Three different mutations at positions 2058 and 2059 (*Escherichia coli* numbering) in region V of the 23S rRNA gene were found. Of the 9 patients with specimens obtained before and after treatment, only 2 had an initial specimen in which the mutation was present, indicating that drug resistance was induced as the result of an inappropriate dosage of azithromycin.

**Conclusion.** Development of macrolide resistance was shown to correlate with subsequent azithromycin treatment failure. The genetic basis for the drug resistance was shown to be mutations in region V of the 23S rRNA gene, which is well described in other Mollicutes. These findings raise concern about the use of single-dose azithromycin treatment of nongonococcal urethritis of unknown etiology.

*Mycoplasma genitalium* is a common cause of acute and chronic nongonococcal urethritis (NGU), primarily in patients without *Chlamydia trachomatis* infection [1]. It is also associated with female cervicitis [2], urethritis [3], and endometritis [4] and has been detected in a tubal specimen from a woman with laparoscopically confirmed salpingitis [5].

In vitro susceptibility studies have thus far shown that all *M. genitalium* isolates tested are highly susceptible to macrolides, with azithromycin having the lowest MIC; quinolones and tetracyclines have been shown to be less active against *M. genitalium* (with the exception of moxifloxacin, which appears to be active against all of the strains examined) [6]. Treatment trials have shown that doxycycline is inefficient in eradicating the infection [7–9], whereas a 5-day course of azithromycin eradicates the bacterium from 95% of patients [9]. Azithromycin, administered as a single 1-g dose given immediately, seems to be less efficient, eradicating the bacterium from ~85% of patients, although this difference did not reach statistical significance [8, 9]. In a study from Australia, however, 28% of the patients experienced relapse or had persistent urethritis after treatment with azithromycin (a single 1-g dose given...
immediately) [10]. Of the patients who experienced azithromycin treatment failure, 5 (56%) of 9 had sexual partners from Southeast Asia, compared with 6 (26%) of 23 of the patients with infections that were responsive to azithromycin treatment. Treatment with moxifloxacin cured all of the patients who experienced azithromycin treatment failure [10]. Specimens obtained from 4 of the Australian patients who experienced treatment failure were collected for culture when the patients attended the sexually transmitted diseases clinic for a test of cure, and 3 specimens were collected from similar patients in Scandinavia. The 7 specimens yielded macrolide-resistant *M. genitalium* isolates with distinct MgPa 1–3 types [11], which indicated that each of the strains was unique. The aim of the study was to establish the reason for the occasional treatment failure.

**PATIENTS, MATERIALS, AND METHODS**

**Patients and specimens.** A case-control study of NGU was conducted from March 2004 through March 2005 at Melbourne Sexual Health Centre, Australia [12]. In this study, 9 men were identified who had experienced treatment failure with a single 1-g dose of azithromycin given immediately, who had no risk of reinfection, and for whom pretreatment and posttreatment urine specimens were available for PCR-based studies. Posttreatment urethral swab specimens were collected specifically for isolation of *M. genitalium* from 4 of these men. Three patients who attended sexually transmitted diseases clinics in Oslo, Norway, or Stockholm and Kristianstad, Sweden, for treatment of NGU and who experienced azithromycin treatment failure were also included; 2 of them had earlier specimens available. Treatment history and the results of *M. genitalium* isolation attempts are summarized in table 1. Patients underwent clinical examination and had first-void urine specimens analyzed for *C. trachomatis* according to the standard procedure of the local laboratory and examined for *M. genitalium* by PCR. Patients in Melbourne, Australia, had urine specimens tested for *M. genitalium* with use of a real-time PCR assay targeting the 16S ribosomal RNA (rRNA) gene, as described by Yoshida et al. [13], whereas urine and swab specimens from the Scandinavian patients were examined with use of a conventional PCR with an internal control for inhibition that targeted the 16S rRNA gene [14]. All positive results were confirmed with use of an independent assay that targeted the MgPa gene (*mgyB*) [15]. Men with *M. genitalium* infection received instruction regarding partner notification and the risk of reinfection, and they were asked to return for a test-of-cure visit 4–6 weeks after treatment. Specimens for culture of *M. genitalium* were urethral swab samples transported in SP4 mycoplasma broth medium. Specimens from Australia were transported on dry ice, whereas specimens from Scandinavia were sent by regular mail to Statens Serum Institut (Copenhagen, Denmark), and growth of *M. genitalium* in Vero cell cultures was performed essentially as described elsewhere [16, 17]. Local ethics committees approved the studies from which the patients were recruited.

**M. genitalium strains studied.** In addition to the 7 *M. genitalium* strains that were isolated from patients who did not respond to azithromycin treatment, 7 genetically distinct strains that were previously isolated at Statens Serum Institut were included in the study (table 2). These strains were highly susceptible to azithromycin and were included for comparison.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed by growing *M. genitalium* in the presence of different concentrations of antibiotics in Vero cell culture. Growth inhibition was monitored by a quantitative TaqMan PCR, as described elsewhere [6, 18].

MICs were determined for erythromycin (Sigma), azithromycin (Groton Laboratories, Pfizer), clarithromycin (Abbott Laboratories), doxycycline (Sigma), tetracycline (Fluka), ciprofloxacin (Bayer Health Care), levofloxacin (Daiichi Sankyo Pharmacology), and moxifloxacin (Bayer Health Care).

**Analysis of genes mediating macrolide resistance.** Two overlapping fragments of the *M. genitalium* 23S rRNA gene were amplified using primers Mg 23S-1992f (5′-TGAAATCCAGGGTGTAAGAC-3′) with Mg 23S-2682r (5′-CGGTCC-TCTCGTGACTAGAACCAAG-3′) and Mg 23S-1986f (5′-GTGTGAAATCTCTTGACTG-3′) with Mg 23S-2171r (5′-TTACATCAAACAAATCCTGCG-3′). A 670–base pair fragment containing the complete 4 L4 gene was amplified with primer Mg L4-4f (5′-AAGTAATGGGCTAACATTAAGTAA-TCC-3′) and Mg L4+1r (5′-TTTAAGAGTAGTGTTGTTGATCATCCATAG-3′). A 560–base pair fragment containing the complete *M. genitalium* L22 gene was amplified with Mg L22-70f (5′-ATGGTAGGCTACAATGGTGAGTTT-3′) and Mg L22+55r (5′-AGTTCTTATTATGCCAACATGCCC-3′).

The amplified fragments were purified and sequenced using ABI Big Dye terminator sequencing kit, version 1.1, and the sequences were read on an ABI 3100 sequencer (Applied Biosystems). The resulting sequences were analyzed and compared with the *M. genitalium* G37 sequence using BioNumerics software, version 4 (Applied Maths).

**Development of a PCR for detection of mutations in the 23S rRNA gene conferring macrolide resistance.** Primers targeting unique regions of the *M. genitalium* 23S rRNA gene flanking the mutations found in region V of the 23S rRNA gene were designed after alignment with sequences from *Mycoplasma pneumoniae*, *Ureaplasma urealyticum*, and other closely related species to produce a 147–base pair amplicon. The primers Mg23S-1992f (5′-CCATCCTGTGCTGTTGCTGCTGCT-3′) and Mg23S-2138r (5′-CCATCCTGCTGCTGCTGCTGCTGCT-3′) were used in a conventional PCR with a 100-μL final reaction volume containing 1X PCR buffer.
Table 1. Treatment history and isolation status of *Mycoplasma genitalium* strains isolated from patients with nongonococcal urethritis enrolled in a study of azithromycin treatment failure.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Strain designation</th>
<th>Origin*</th>
<th>23S rRNA genotype before treatment</th>
<th>Prescribed treatment</th>
<th>PCR result after treatment and 23S rRNA genotype</th>
<th>Isolation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M 6271</td>
<td>Melbourne, Australia (case 1)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Azm 1 g stat; (3) Azm 1 g weekly for 3 weeks; (4) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, NA; (2) positive, NA; (3) positive, A2058G; (4) negative</td>
<td>Growth in Vero cells only</td>
</tr>
<tr>
<td>2</td>
<td>M 6270</td>
<td>Melbourne, Australia (case 2)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Azm 1 g stat; (3) Azm 1 g weekly for 3 weeks; (4) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, NA; (2) positive, NA; (3) positive, A2058G; (4) negative</td>
<td>Isolation in Friis’ modified mycoplasma broth medium</td>
</tr>
<tr>
<td>3</td>
<td>M 6320</td>
<td>Melbourne, Australia (case 3)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Azm 1 g weekly for 3 weeks; (3) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, NA; (2) positive, A2058G; (3) negative</td>
<td>Isolation in Friis’ modified mycoplasma broth medium</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>Melbourne, Australia (case 4)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, A2059G; (2) negative</td>
<td>No specimen collected for culture</td>
</tr>
<tr>
<td>5</td>
<td>M 6321</td>
<td>Melbourne, Australia (case 5)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, A2058G; (2) negative</td>
<td>Isolation in Friis’ modified mycoplasma broth medium</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>Melbourne, Australia (case 6)</td>
<td>A2058G</td>
<td>(1) Azm 1 g stat; (2) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, A2058G; (2) negative</td>
<td>No specimen collected for culture</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>Melbourne, Australia (case 7)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Mox 400 mg daily for 10 days</td>
<td>(1) Negative; (2) negative</td>
<td>No specimen collected for culture</td>
</tr>
<tr>
<td>8</td>
<td>NA</td>
<td>Melbourne, Australia (case 8)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Mox 400 mg daily for 10 days</td>
<td>(1) Negative; (2) negative</td>
<td>No specimen collected for culture</td>
</tr>
<tr>
<td>9</td>
<td>NA</td>
<td>Melbourne, Australia (case 9)</td>
<td>WT</td>
<td>(1) Azm 1 g stat; (2) Mox 400 mg daily for 10 days</td>
<td>(1) Positive, A2059G; (2) negative</td>
<td>No specimen collected for culture</td>
</tr>
<tr>
<td>10</td>
<td>M 6257</td>
<td>Kristianstad, Sweden</td>
<td>NA</td>
<td>(1) Dox 200-mg loading dose and 100 mg daily for 9 days thereafter; (2) Azm 1 g stat; (3) Dox 200-mg loading dose and 100 mg daily for 18 days thereafter; (4) Azm 1 g stat and 500 mg daily for 6 days thereafter; (5) Dox 200 mg daily for 15 days; (6) Mox 400 mg daily for 10 days;</td>
<td>(1) Positive, WT; (2) positive, NA; (3) positive, A2058G; (4) positive, A2058G; (5) NA; (6) negative</td>
<td>Isolation in Friis’ modified mycoplasma broth medium</td>
</tr>
<tr>
<td>11</td>
<td>M 6302</td>
<td>Stockholm, Sweden</td>
<td>NA</td>
<td>(1) Dox 200-mg loading dose and 100 mg daily for 9 days; (2) Azm 500-mg loading dose and 250 mg daily for 4 days; (3) Dox 200 mg daily for 15 days; (4) Azm 1 g weekly for 3 weeks; (5) Mox 400 mg daily for 10 days;</td>
<td>(1) Positive, NA; (2) positive, NA; (3) positive, A2058G; (4) positive, A2058G; (5) negative</td>
<td>Growth in Vero cells only</td>
</tr>
<tr>
<td>12</td>
<td>M 6303</td>
<td>Oslo, Norway</td>
<td>A2059G</td>
<td>(1) Azm 1 g stat; (2) Azm 1 g stat; (3) Azm 500-mg loading dose and 250 mg daily for 4 days thereafter; (4) Dox 200 mg daily for 15 days</td>
<td>(1) NA; (2) positive A2059G; (3) positive A2059G; (4) NA</td>
<td>Isolation in Friis’ modified mycoplasma broth medium</td>
</tr>
</tbody>
</table>

**Note.** (1), First-line treatment; (2), second-line treatment; (3), third-line treatment; (4), fourth-line treatment; (5), fifth-line treatment; Azm, azithromycin; Dox, doxycycline; Mox, moxifloxacin; NA, not available; WT, wild-type sequence.

*Case numbering of Australian specimens was according to Bradshaw et al. [10].*
Table 2. MICs for selected antimicrobial agents, as determined by the cell culture method, for 7 strains of *Mycoplasma genitalium* isolated from patients with nongonococcal urethritis who experienced azithromycin treatment failure and 7 susceptible strains.

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Erythromycin</th>
<th>Azithromycin</th>
<th>Clarithromycin</th>
<th>Doxycycline</th>
<th>Tetracycline</th>
<th>Ciprofloxacin</th>
<th>Levofloxacin</th>
<th>Moxifloxacin</th>
<th>Rpl-4</th>
<th>Rpl-22</th>
<th>23S rRNA</th>
<th>Gene mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6257</td>
<td>≥16</td>
<td>≥8</td>
<td>≥16</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>A2058G</td>
</tr>
<tr>
<td>M6270</td>
<td>≥16</td>
<td>≥8</td>
<td>≥16</td>
<td>0.25</td>
<td>0.5</td>
<td>4</td>
<td>2</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td>E123K A2058G</td>
</tr>
<tr>
<td>M6271</td>
<td>≥16</td>
<td>≥8</td>
<td>≥16</td>
<td>0.25</td>
<td>0.25</td>
<td>4</td>
<td>1</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td>A2058C</td>
</tr>
<tr>
<td>M6302</td>
<td>≥16</td>
<td>≥8</td>
<td>≥16</td>
<td>0.125</td>
<td>0.5</td>
<td>4</td>
<td>1</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td>A2058G</td>
</tr>
<tr>
<td>M6303</td>
<td>≥16</td>
<td>≥8</td>
<td>≥16</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>H69R A2059G</td>
</tr>
<tr>
<td>M6320</td>
<td>≥64</td>
<td>≥32</td>
<td>≥16</td>
<td>0.25</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.063</td>
<td></td>
<td></td>
<td></td>
<td>A2059G</td>
</tr>
<tr>
<td>M6321</td>
<td>≥64</td>
<td>≥32</td>
<td>≥16</td>
<td>0.125</td>
<td>0.25</td>
<td>2</td>
<td>0.5</td>
<td>0.063</td>
<td></td>
<td></td>
<td></td>
<td>A2058G</td>
</tr>
<tr>
<td>M2300</td>
<td>0.125</td>
<td>0.008</td>
<td>0.031</td>
<td>0.5</td>
<td>0.25</td>
<td>16</td>
<td>4</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>M2321</td>
<td>0.063</td>
<td>0.004</td>
<td>0.016</td>
<td>1</td>
<td>0.5</td>
<td>8</td>
<td>4</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>M2341</td>
<td>0.25</td>
<td>0.008</td>
<td>0.031</td>
<td>0.25</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>M6282</td>
<td>0.125</td>
<td>0.008</td>
<td>0.063</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>M6287</td>
<td>0.125</td>
<td>0.016</td>
<td>0.031</td>
<td>0.25</td>
<td>0.25</td>
<td>8</td>
<td>2</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>M6280</td>
<td>0.063</td>
<td>0.004</td>
<td>0.016</td>
<td>0.125</td>
<td>0.25</td>
<td>1</td>
<td>0.5</td>
<td>0.063</td>
<td></td>
<td></td>
<td></td>
<td>N172S</td>
</tr>
<tr>
<td>M6151</td>
<td>0.125</td>
<td>0.008</td>
<td>0.031</td>
<td>0.25</td>
<td>0.25</td>
<td>4</td>
<td>1</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
</tbody>
</table>

*NOTE.* WT, wild-type sequence.
(PlatinumTaq; Invitrogen) consisting of 20 mmol/L tris-hydrochloride with a pH of 8.4 and 50 mmol/L potassium chloride, with 1.5 mmol/L magnesium chloride; 0.4 mmol/L of each primer; 125 μmol/L each of dATP, dGTP, and dCTP and 250 μmol/L dUTP; and 2 U of Platinum Taq DNA polymerase (Invitrogen). After activation of the enzyme at 95°C for 2 min, 40 cycles each consisting of a 15-s denaturation at 95°C and a 60-s combined annealing and extension step at 60°C were performed. Positive specimens were sequenced in both directions with the same primers that were used for the amplification.

To evaluate the specificity of the mutation detection assay, a panel of 100 urogenital specimens that were randomly selected from specimens submitted for routine C. trachomatis PCR testing was subjected to the mutation detection PCR. All of them were found to be negative for M. genitalium by TaqMan PCR [18]. Respiratory tract specimens obtained from 10 patients with M. pneumoniae infection as documented by PCR [19], as well as a purified DNA preparation of M. pneumoniae containing 1 × 10^4 DNA copies, were also tested in the mutation detection assay. Purified M. genitalium G37 DNA was used as a template in the mutation detection assay and was diluted to <10 genome copies to demonstrate the limit of detection of the assay.

**Genotyping of M. genitalium in pretreatment and post-treatment specimens.** M. genitalium in pretreatment and posttreatment specimens from the 9 Australian patients and from 2 of the Scandinavian patients, as well as the strains isolated in the Vero cell culture system, were genotyped using the M. genitalium MgPa 1–3 typing system, as described elsewhere [11]. In brief, specimens were amplified and then sequenced using the MgPa-1 and MgPa-3 primer set described elsewhere [15]. In 2 of the 9 Australian specimen sets, the posttreatment specimen did not amplify; therefore, only the pretreatment specimen sequence was available.

**RESULTS**

**Isolation of M. genitalium from men who experienced azithromycin treatment failure.** M. genitalium was propagated in the Vero cell culture system from all 7 specimens for which culture was attempted. Five of the strains were subsequently adapted to grow in Friis’ modified mycoplasma medium; 4 were single-colony cloned for 3 rounds to ensure homogeneity of the strains, and the remaining strain is, to date, still in the process of being single-colony cloned. Two strains appeared to be unable to grow in axenic medium.

MICs could be determined in the cell culture system for all 7 strains, and all of the strains were highly resistant to macrolides (both 14-membered and 15-membered macrolides); the MIC of azithromycin was >8 μg/mL. Individual MICs for all strains are shown in table 2.

**Analysis of genes mediating macrolide resistance in 14 M. genitalium strains.** Point mutations were found in the L4 and L22 genes, respectively, but most of these did not lead to amino acid changes. In L4, an H69R amino acid substitution was found in strain M6303. This was the only amino acid change in regions previously shown to be involved in macrolide resistance in mollicutes [20]. In the macrolide-susceptible strain M 6280, an N172S substitution was found in L4, and in the macrolide-resistant strain M 6270, an E123K substitution was found in L22 (table 2). These 2 mutations, however, have not previously been associated with drug resistance.

No mutations were detected in region II of the 23S rRNA gene, but 3 different mutations were detected in region V. An A2058G transition (Escherichia coli numbering) was found in 3 of the resistant strains, whereas an A2059G transition in the adjacent position was detected in 3 other strains. One strain had an A2058C transversion. All 3 mutations led to high-level macrolide resistance to both 14-membered and 15-membered macrolides (table 2).

**PCR for detection of mutations in the 23S rRNA gene that confer macrolide resistance.** To evaluate the specificity of the PCR detecting the mutations in positions 2058 and 2059 (E. coli numbering) of the 23S rRNA gene, a panel of 100 urogenital specimens that were randomly selected from specimens submitted for routine C. trachomatis testing was subjected to the PCR. All of the samples had previously been found to be negative for M. genitalium by TaqMan PCR [18], and none of the samples reacted in the mutation detection assay. Respiratory tract specimens obtained from 10 patients with M. pneumoniae infection documented by PCR also had negative results in the mutation detection PCR, as did a purified DNA preparation of M. pneumoniae that contained 1 × 10^4 DNA copies per PCR reaction. With use of purified M. genitalium G37 DNA as a template, the mutation detection assay produced clear results, even with an input of <10 genome copies, further demonstrating its applicability for detecting the mutations directly in clinical specimens.

The assay was applied to pretreatment and posttreatment specimens obtained from 9 patients who were enrolled in Australia and 2 patients who were enrolled in Scandinavia. However, complete sequence sets were obtained from only 9 patients, because 2 of the posttreatment specimens from the Australian patients did not amplify. Surprisingly, only 2 patients had strains with an A2058G or A2059G mutation (1 patient each) detected in the pretreatment specimens, suggesting that, in the majority of the patients, the drug-resistant mutant was selected for during treatment. The fact that the MgPa 1–3 type of the M. genitalium strains that were present in the pretreatment and posttreatment specimens were identical strongly suggests that the patients were not coinfected with 2 M. genitalium strains, with subsequent selection of the drug resistant type.
DISCUSSION

In the present study, we isolated *M. genitalium* from 7 men who experienced failure of treatment with azithromycin. We found that mutations at 2 positions in region V of the 23S rRNA gene of *M. genitalium* were strongly associated with resistance to both 14-membered macrolides, such as erythromycin and clarithromycin, and 15-membered macrolides, such as azithromycin. The level of drug resistance was high, leading to failure of therapy regardless of dosage. The resistance appeared to be efficiently induced by insufficient therapy with azithromycin administered as a single 1-g dose given immediately, but it occurred in strain M6302 even after treatment with the extended azithromycin dosage scheme (a single 500-mg dose administered on day 1 and 250-mg doses administered on days 2–5) that is currently recommended for treatment of *M. genitalium* infection in Scandinavia. Whether this reflects that the patient had acquired a drug-resistant strain initially or whether the drug resistance was induced during treatment cannot be determined because of the lack of a pretreatment specimen.

Naturally occurring macrolide resistance has not, to our knowledge, previously been reported for *M. genitalium*, and in vitro selection has only been reported for the closely related species *M. pneumoniae*. Using an in vitro selection system, Pereyre et al. [20] reported the selection of macrolide-resistant mutants with mutations primarily occurring in the 23S rRNA gene. The mutation selected by erythromycin and azithromycin was C2611A (*E. coli* numbering) in region V of the 23S rRNA gene, in positions different from the 2058/2059 positions found in clinical isolates and those induced in vitro in other studies [21, 22]. By selection with josamycin and quinupristin-dalfopristin, an A2062G mutation was induced. The A2062G and C2611A mutations alone generally led to only moderately elevated MICs. This is in contrast with the high-level resistance resulting from mutations in the 2058/2059 positions.

Although mutations in the L4 and L22 genes have been reported to confer macrolide resistance in other mollicutes, we found only 1 amino acid substitution in L4 in a region previously described to confer low-grade resistance in *M. pneumoniae* strains selected by growth in increasing concentrations of antibiotic [20]. Although several point mutations were observed, most of them did not result in amino acid changes and could not explain the resistance.

Other mechanisms, such as methylation of the macrolide binding site or the presence of efflux pumps, both of which mediate macrolide resistance in other bacteria [23], were not investigated in the present study. Because of their absence in other mollicutes and the 100% agreement between in vitro macrolide resistance, failure of azithromycin therapy, and the presence of mutations identified in region V of the 23S rRNA gene, it seems unlikely that they play a role.

Macrolide resistance in *M. pneumoniae*, the closest relative of *M. genitalium*, has recently been demonstrated to be a dramatically increasing problem in strains isolated from pediatric patients in Japan; the prevalence of macrolide resistance has increased from 0% of such strains in 2002 to 31% in 2006 [24]. It was, therefore, not surprising that Bradshaw et al. [10] found that 56% of *M. genitalium*-positive patients with NGU who experienced azithromycin treatment failure had acquired their infection in Southeast Asia, compared with 27% of those with a favorable response to azithromycin. However, the finding that only 1 of the 9 Australian pretreatment specimens included in this study carried a strain with the macrolide resistance mutation in the 23S rRNA gene questions the importance of this finding. There is no evidence indicating that the macrolide-resistant strains have a slower growth rate in vitro or in vivo. The rapid spread of the macrolide-resistant *M. pneumoniae* strains also suggests that the fitness of the mutated strains is not significantly impaired.

Temporary subjective clinical relief was experienced by 8 of the 9 Australian patients during azithromycin treatment, whereas 1 patient was persistently asymptomatic. Interestingly, the 1 patient in whom a strain with the resistance mutation was detected in the pretreatment specimen also reported improvement during azithromycin treatment. This may be attributable to the immunomodulating properties of azithromycin [25] and raises interesting questions regarding the possibility of treating recurrent pathogen-negative NGU with nonsteroidal anti-inflammatory drugs. Indeed, in a previous study involving men with acute NGU, it was shown that recurrences were fewer among those patients who received a combination of ketoprofen and doxycycline [26]. These findings, however, need to be confirmed in larger, well-controlled studies.

The use of the Vero cell culture system for initial propagation of *M. genitalium* strains from clinical specimens and the subsequent direct determination of MICs in the cell culture [6] clearly demonstrated the potential of this method for further studies of the clinically relevant breakpoints for various antibiotics. All 7 strains were capable of multiplying in the cell culture system. Although some of the specimens contained a very low number of DNA copies, as determined by the quantitative TaqMan PCR assay, the organisms grew relatively readily, and MIC determinations could generally be performed within a 2–3-month period. Although this may seem to be very slow, it is still a significant reduction in the time required to obtain results, compared with the time needed for adaptation of the mycoplasmas to growth in axenic culture. Furthermore, the use of the Vero cell culture system is even more important, because some of the strains could not be adapted to axenic growth, even after repeated attempts for >1 year. It is obvious, however, that culture-based methods are of little help in the
treatment of patients, and therefore, the development of the mutation detection assay represents a major improvement. Although sequencing of the amplified region V fragment was straightforward, it might be possible to simplify the assay further by applying a simple restriction enzyme digestion, as described elsewhere for \textit{M. pneumoniae} [27].

The mutation detection assay showed no cross-reactions with \textit{M. pneumoniae} when purified DNA was used in a relatively high concentration. None of the clinical specimens obtained from patients with \textit{M. pneumoniae} infection reacted in the mutation detection assay. However, if very large amounts of \textit{M. pneumoniae} DNA were present, slight cross-reactions could not be excluded. Although \textit{M. pneumoniae} has been detected in the urogenital tract [28, 29], it is not commonly found at this site [30], and amplicons from \textit{M. pneumoniae} could eventually be distinguished from amplicons from \textit{M. genitalium} by a single-nucleotide difference in the amplified sequence. The finding that all of the 100 \textit{M. genitalium}–negative urogenital specimens reacted negatively in the mutation detection assay provides confidence in the results from \textit{M. genitalium}–positive specimens.

In the present study, we have shown that macrolide resistance is occurring in a small proportion of patients who are initially treated with azithromycin. Furthermore, the mutation detection assay that we have developed could be a valuable tool in directing appropriate treatment of these patients. Second-line treatments for \textit{M. genitalium} have not been extensively studied, and although moxifloxacin appears to eradicate infection in patients who experience azithromycin failure, it is costly, and the risk of developing quinolone resistance is considerable if this treatment is used inappropriately. The results of the present study indicate that it would not be possible to predict azithromycin treatment failure in all patients at first presentation, and the mutation detection assay may be best used for patients who experience macrolide treatment failure. Because azithromycin has been recommended as a presumptive treatment for NGU and for \textit{M. genitalium} infection in many countries, we may expect treatment failure to be increasingly reported. These findings have direct application to clinical practice and highlight the need for further studies to determine optimal first-line and second-line treatments for NGU in general and for \textit{M. genitalium} infection in men and women in particular.

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