Mycoplasma genitalium and Macrolide Resistance-associated Mutations in the Skåne Region of Southern Sweden 2015

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***Mycoplasma genitalium* is a sexually transmitted infection ordinarily treated with azithromycin. Emerging resistance to macrolide is linked to mutations in the 23S rRNA gene. We analysed the frequency of such mutations of *M. genitalium* isolates from patients that were symptomatic, and from sexual partners of symptomatic individuals, from October to December of 2015, in the Skåne Region of Sweden. Mutations were analysed by the use of DNA sequencing. Overall, 11.9% (145/1,311) and 17.0% (116/704) of females and males were positive for *M. genitalium*, respectively. Macrolide resistant mutations were detected in 13% (31/239) of *M. genitalium* isolates from first-test patient samples. Twenty-one (8.8%) and 10 (4.2%) of the isolates had point mutations of the 23S-gene at position 2072 and 2071, respectively. Two different *M. genitalium* isolates were detected simultaneously in two cases. In summary, we found a relatively low rate of macrolide-resistant *M. genitalium* in the region of Southern Sweden.**

Key words: mycoplasma genitalium; macrolide; azithromycin; resistance.

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Mycoplasma genitalium was discovered from men with non-gonococcal urethritis (NGU) in 1981 (1). *M. genitalium* is a sexually transmitted bacterium and is a major cause of NGU in males (2). It is also associated with female cervicitis and complications such as pelvic inflammatory disease (PID) (3). The macrolide azithromycin is commonly used for treatment of *M. genitalium*. Mutations in region V of the 23S rRNA gene of *M. genitalium* are consistently linked to macrolide resistance and were first described in 2008 by Jensen et al. (4). However, emerging resistance problem against *M. genitalium* have been reported in Scandinavia, where a Swedish study observed an increase in macrolide resistance before introduction of treatment from 0% in 2006 to 18% in 2011 (5), and a Denmark survey detected macrolide resistance in 38% of *M. genitalium*-positive unselected patients (6). Recently, a Swedish study reported macrolide resistance of 18% among *M. genitalium* isolates collected throughout 2012 in the Stockholm region (7). The aim of the present study was to investigate the prevalence of macrolide-associated mutations among *M. genitalium* isolates in the Skåne Region within the 4th quarter of 2015.

MATERIALS AND METHODS

Study population

Within the region of Skåne Sweden with 1.3 million habitants[1] urogenital samples, from 3,167 males (mean age 32 years, median 29 years, range 13–82 years) and 5,636 women (mean age 28 years, median 26 years, range 2–77 years) who were seeking care at diverse clinics, such as sexual health clinics, youth clinics and general practitioners, were routinely tested for *M. genitalium* during 2015. According to Swedish guidelines, urogenital samples sent for *M. genitalium* diagnostics were from patients with clinical symptoms related to *M. genitalium* and from asymptomatic sexual partners to *M. genitalium* positive patients. The samples were analysed for *M. genitalium* at the Clinical Microbiology Laboratory, Region Skåne, Sweden by the use of a real-time PCR (8).

Study design

A retrospective study of macrolide-resistance was performed on *M. genitalium* isolates collected from October to December 2015. The *M. genitalium* isolates were originally detected in urogenital samples by routine testing by the use of real-time PCR (8). A sample was classified as a first-test patient sample if no previous sample had been collected within the preceding 6 weeks. The length of 6 weeks was chosen in order to include relatively recently acquired infections among the first-test patient samples. This period is in agreement with that of the new European guidelines from 2016 where samples for test of cure should be collected no earlier than 3 weeks after start of treatment (9).

Laboratory procedures

From samples positive for *M. genitalium*, remaining materials of nucleic acid extractions (Magna LC, Total NA. Large Volume kit, 500 ul input 50 ul output) from APTIMA Urine/Vaginal/Swab collection tubes were stored at –20°C. For amplification of the *M. genitalium* 23S rRNA gene of region V, we used a 20 µl reaction of 1 x Perfecta™ qPCR Fast Mix™ Low Rox, (Quanta Biosciences, Gaithersburg, Maryland, USA), 0.125% (w/v) BSA (Ultrapure™, Ambion, Thermofisher Scientific, MA, USA), 0.2 µm of each forward (Mg 23S-1992F) and reverse primer (Mg 23S-2138R) (4) (LGC Biosearch Technologies, Risskov, Denmark) and 5 µl of nucleic acid extraction from each sample. The PCR reaction was carried out in an automated thermocycler (ABI 9700) as follows; 20''at 95°C and then 50 cycles of 3'' at 95°C and 1' at 60°C. Purified DNA of *M. genitalium* (Amplirun® Mycoplasma genitalium

[1]"Population in the country, counties and municipalities, 31 December 2015". Central Bureau of Statistics. Read February 23, 2016.

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DNA control, Vircell, Granada, Spain) was included as a positive control and water was used as a negative control in each run. The sensitivity of the PCR was about 70 copies/PCR by the use of the *M. genitalium* DNA control (Vircell, Granada, Spain).

In order to visualise the amplicon of the correct size (147 bp) an automated capillary electrophoresis device was used (QIAxcel system and Screen Gel Software, Qiagen Hilden, Germany). Briefly, post PCR samples in a 12-well strips were loaded and separation (about 4' for 12 samples) was performed with alignment marker QX of 15 to 3,000 bp. If the amplicon was not detected, PCR and electrophoresis was repeated once and the result was considered final.

For detection of macrolide-associated mutations the amplified fragment were purified by illustra MicroSpin S-300 HR (GE Healthcare Life Science, Fairfield, USA) and one strand was amplified using the forward primer (Mg 23S-1992F) and the ABI Big Dye terminator sequencing kit, version 3.1 (ABI, Foster City, California, USA) in a thermocycler (TPersonal, Biometra, Göttingen, Germany). The amplicon was purified by a NucleoSEQ® Dye-terminal column (Macherey-Nagel, Düre, Germany) and sequence were read on an ABI 3130 Genetic analyser (Applied Biosystems). The sequences were compared with the 23S ribosomal RNA gene of the *M. genitalium* G-37 isolate (GenBank accession number NR_077054.1) using BioEdit Sequence Alignment Editor v7.0 (10). Identified mutations were given positions according to the 23S ribosomal RNA gene of the *M. genitalium* G-37 isolate (GenBank accession number NR_077054.1) where positions 2071 and 2072 correspond to that of positions 2058 and 2059 of *E. coli*, respectively. Overall, 271 samples generated successful DNA sequences for mutation analysis.

All electropherograms were inspected visually. Only DNA sequences of high quality, characterised by sharp peaks and little to no background were subjected to alignment. To validate our assay, we analysed 5 *M. genitalium*-positive samples with known status of the 23S ribosomal RNA gene (4 samples with mutant isolates and one sample with a wild type isolate), that had been previously analysed by another laboratory. All results showed complete agreement between our laboratories (Table SI[2]).

During October through December 2015, 307 samples were positive for *M. genitalium* according to our in-house PCR, and 280 samples (91%) were available for analysis of macrolide-associated resistance mutations. Amplification of the 23S rRNA gene prior to sequence analysis was successful for 99% (276/280) of the samples as visualized by electrophoresis (QIAxcel, Qiagen), and 98% (271/276) of these samples generated successful DNA sequences for mutation analysis.

Ethical consideration

In accordance with Swedish law on development projects, data were anonymised after extraction from the patient records. No actions or treatments of patients were changed from routine management.

RESULTS

In our study we used of urogenital samples from people that were symptomatic, and from the sexual partners of symptomatic individuals. During October through December 2015, 11.9% (145/1311) and 17.0% (116/704) of females and males were positive for *M. genitalium*, respectively.

Overall, 82% (271/332) of samples positive for *M. genitalium* were successfully analysed for macrolide-associated resistance mutations in the 23S rRNA gene. Among the "first-test" patient samples, 13% (31/239) manifested mutations in the 23S rRNA gene (**Table I**). We also detected two different *M. genitalium* isolates simultaneously in two cases (Table I).

Among 32 samples categorised as second, third or fourth serial specimens from the patients the corresponding mutation rates were 83% (20/24), 100% (7/7) and 100% (1/1), respectively (Table I). For 15 patients, mutation signatures were obtained for serial samples positive for *M. genitalium*. Notably, for 5 of these patients (33%) we initially detected the wild type *M. genitalium* whereas mutations in the 23S rRNA gene were present in the follow-up sample (4 patients with A2072G and one patient with A2071G) (**Table II**). Nine patients demonstrated identical mutations in the initial and follow-up sample (Table II).

DISCUSSION

Among the first-test patient samples in the region of Skåne, 13% of *M. genitalium* isolates manifested mutations linked to macrolide resistance. This is similar to a study from France (14%) (11), and to other regions of Sweden such as Stockholm and Dalarna (both 18%) (5, 7). However, it is clearly different from that of Denmark with a higher proportion of mutated *M. genitalium* isolates (38%) (Fisher's exact test, $p<0.0001$) (6). Thus, although Skåne has been connected with Denmark by the Öresund bridge since the year 2000, the Skåne region appears to have a lower rate of macrolide-associated mutations among *M. genitalium* isolates. In Sweden the first line of treatment of *C. trachomatis* is doxycycline. This is different from Denmark and Greenland where azithromycin is the first line of treatment for *C. trachomatis* and NGU. In Greenland, macrolide resistance rates of 100% in *M. genitalium* isolates have been reported (12). It is plausible that the high Danish macrolide resistance rates can be largely explained by this factor. Another important factor is the extended azithromycin regime for

Table I. Frequency of wild type (WT) and point mutations of 23S gene of *M. genitalium* (M.g) isolates

Mutation profile	First-test sample[a] *n* (%)	Second sample[b] *n* (%)	Third sample *n* (%)	Fourth sample *n* (%)	Total *n* (%)
WT	208 (87)	4 (17)	– (–)	– (–)	212 (78)
A2071G[c]	8 (3.3)	10 (42)	5 (71)	– (–)	23 (8.4)
A2072G	20 (8.4)	10 (42)	2 (29)	1 (100)	33 (12)
A2072C	1 (0.4)	– (–)	– (–)	– (–)	1 (0.4)
2071T/G	1 (0.4)	– (–)	– (–)	– (–)	1 (0.4)
WT+A2071G	1 (0.4)	– (–)	– (–)	– (–)	1 (0.4)
Samples with mutated M.g	31 (13)	20 (83)	7 (100)	1 (100)	59 (22)
Total	239	24	7	1	271

[a]First-test patient sample where the patient had no sample analysed within the previous 6 weeks. [b]Second sample collected from patients within the study period. [c]Position numbers are according to the 23S ribosomal RNA gene of the *M. genitalium* G-37 isolate (GenBank accession number NR_077054.1).

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Table II. Mutation profile of r23S gene among serial samples of 15 patients with *M. genitalium*

Pat. No.	Date of sample collection dd/mm/year	Sex	Source of specimen	*M. genitalium* PCR. Mean Ct	Mutation
1	05-11-2015	F	Vaginal swab	29.1	WT
	02-12-2015		Vaginal swab	34.8	A2072G[a]
2	03-06-2015	F	Vaginal swab	38.7	Not analysed
	23-09-2015		Vaginal swab	33.7	Not analysed
	16-11-2015		Vaginal swab	26.4	A2071G
	16-12-2015		Vaginal swab	28.2	A2071G
3	10-11-2015	F	Vaginal swab	35.2	A2071G
	27-11-2015		Vaginal swab	31.9	A2071G
4	28-09-2015	M	First-void urine	32.1	Not analysed
	16-10-2015		First-void urine	32.1	A2071G
	30-10-2015		First-void urine	31.1	A2071G
5	12-10-2015	M	First-void urine	34.8	A2071G
	22-10-2015		First-void urine	29.9	A2071G
6	14-10-2015	M	First-void urine	26.3	WT
	20-11-2015		Urethral swab	23.1	A2072G
7	30-11-2015	F	Vaginal swab	35.4	WT
	10-12-2015		Vaginal swab	34.2	WT
8	02-10-2015	F	Vaginal swab	26.8	A2072G
	16-10-2015		Vaginal swab	29.7	A2072G
	02-11-2015		Vaginal swab	30.3	A2072G
	09-11-2015		Vaginal swab	30.5	A2072G
9	08-10-2015	M	First-void urine	32.5	A2071G
	10-11-2015		First-void urine	31.8	A2071G
	30-12-2015		First-void urine	23.8	A2071G
10	20-10-2015	F	Vaginal swab	29.0	WT
	25-11-2015		Vaginal swab	36.5	A2071G
	10-12-2015		Vaginal swab	26.8	A2071G
11	06-10-2015	M	First-void urine	28.4	WT
	24-10-2015		First-void urine	31.1	A2072G
12	03-11-2015	M	First-void urine	29.4	A2071G
	26-11-2015		First-void urine	38.3	A2071G
13	05-11-2015	M	First-void urine	25.2	A2072G
	22-12-2015		First-void urine	31.1	A2072G
14	03-11-2015	M	First-void urine	24.1	WT
	15-12-2015		First-void urine	29.7	A2072G
15	09-10-2015	F	Vaginal swab	34.5	A2072G
	19-11-2015		Vaginal swab	35.5	A2072G

[a]Position numbers are according to the 23S ribosomal RNA gene of the *M. genitalium* G-37 isolate (GenBank accession number NR_077054.1).

5 days for treatment of *M. genitalium* commonly given in Sweden (500 mg on day 1 and then 250 mg daily on days 2, 3, 4 and 5). This might be the reason for the relatively low level of macrolide resistance compared to that of Denmark where single dose 1g azithromycin is usually used. Accordingly, macrolide resistance rates of 30–45% are reported where 1 g single dose azithromycin is widespread (9). In contrast, a recent study did not find reduced macrolide resistance among the extended azithromycin 1.5 g regime compared to the single 1 g dose treatment (13).

However, the 5 day regime of azithromycin is now recommended in the 2016 European guideline on *M. genitalium* infections for uncomplicated *M. genitalium* infections (9).

In our study, two samples with mixed infections, one case with 2071T+2071G and another case with wild type + 2071G, were detected by the Sanger sequencing chromatograms (Figs S1 and S2[2]). In addition, these mixed infections were detectable by an in house real-time PCR for simultaneous detection of wild type and of macrolide-associated mutations of the 23S rRNA gene of *M. genitalium* (Figs S3 and S4[2]). Mixed infections have also been demonstrated in a French study (11). However, individuals with mixed infections of wild type and macrolide-resistant strains should be treated with a second-line antibiotic (e.g. moxifloxacin) in order to minimise selection of the macrolide-resistant strains.

In our region the most common mutation was A→G at position 2072 (8.4%, 20/239) followed by A→G at position 2071 (4.2%, 10/239) of the 23S-gene (Table I). In contrast, the A→G mutation at position 2071 was predominant in Denmark (23%, 232/1008) (6) and in Dalarna in Sweden (25%, 8/32) (5). It is tempting to speculate that the difference in these proportions of the mutated isolates is due to regional spread of mutated clones of *M. genitalium*.

Our study has limitations since 27 DNA extractions of 307 *M. genitalium* isolates were missing, and 4 *M. genitalium* positive samples could not be re-amplified by the 23S-PCR. The reasons for the failure of the 23S-PCR was probably the low amount of *M. genitalium* in the DNA extractions since the mean Ct-value was 38.5 (range 36.0–42.5) among these samples according to the first line PCR-test used for detection of *M. genitalium* (8). Also, one sequencing reaction failed for unknown reasons. Despite these drop outs, we consider the used samples series as representative for the macrolide resistance occurrence among patients who were seeking care in Skåne, since all testing for *M. genitalium* in the Skåne region is performed by our laboratory.

Among the patients with follow-up samples we observed high macrolide-resistance rates of 83% and up to 100% for the third and fourth serially taken sample. Furthermore, among 5 patients only the wild type isolate was detected in the first-test patient samples while mutated isolates where found in the follow-up samples. Although, no data on treatment regime could be obtained due to the design of our study, it is likely that selection for mutated *M. genitalium* occurred due to pressure from azithromycin. Such selection of mutated *M. genitalium* during azithromycin treatment has been reported (4, 11, 14–16). However, our 5 results of wild type isolates in the first-test patient samples and mutated isolates in the follow-up samples may indicate a false negative test of mutated *M. genitalium* in the first samples of these patients. In addition, we cannot exclude the possibility that these patients had a second infection with newly acquired mutant *M. genitalium*.

In conclusion, we report a relatively low rate of macrolide-resistant *M. genitalium* isolates in the region of Skåne in Southern Sweden. In accordance with the 2016 European recommendation (9), our laboratory will implement a macrolide resistance test in order to minimize treatment failures and to limit the spread of macrolide-resistant *M. genitalium*.

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