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Published in:
Clinical Microbiology and Infection

DOI:
10.1016/j.cmi.2017.09.006

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Clinical Microbiology and Infection 24 (2018) 533–539

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Clinical and analytical evaluation of the new Aptima *Mycoplasma genitalium* assay, with data on *M. genitalium* prevalence and antimicrobial resistance in *M. genitalium* in Denmark, Norway and Sweden in 2016

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ARTICLE INFO

Article history:
Received 6 August 2017
Received in revised form
7 September 2017
Accepted 8 September 2017
Available online 18 September 2017

Editor: F. Allerberger

Keywords:
16S rRNA
23S rRNA
Antimicrobial resistance
Aptima
Azithromycin
Hologic
Moxifloxacin
Mycoplasma genitalium
*par*C

ABSTRACT

Objectives: *Mycoplasma genitalium* (MG) causes urethritis and cervicitis, potentially causing reproductive complications. Resistance in MG to first-line (azithromycin) and second-line (moxifloxacin) treatment has increased. We examined the clinical and analytical performance of the new Conformité Européene (CE)/*in vitro* diagnostics (IVD) Aptima *Mycoplasma genitalium* assay (CE/IVD AMG; Hologic); the prevalence of MG, *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG); and MG resistance to azithromycin and moxifloxacin in Denmark, Norway and Sweden in 2016.

Methods: From February 2016 to February 2017, urogenital and extragenital (only in Denmark) specimens from consecutive attendees at three sexually transmitted disease clinics were tested with the CE/IVD AMG, the research-use-only MG Alt TMA-1 assay (Hologic), Aptima Combo 2 (CT/NG) assay and a laboratory-developed TaqMan real-time *mgp*B quantitative real-time PCR (qPCR). Resistance-associated mutations were determined by sequencing. Strains of MG and other mycoplasma species in different concentrations were also tested.

Results: In total 5269 patients were included. The prevalence of MG was 7.2% (382/5269; 4.9–9.8% in the countries). The sensitivity of the CE/IVD AMG, MG Alt TMA-1 and *mgp*B qPCR ranged 99.13–100%, 99.13–100% and 73.24–81.60%, respectively, in the countries. The specificity ranged 99.57–99.96%, 100% and 99.69–100%, respectively. The prevalence of resistance-associated mutations for azithromycin and moxifloxacin was 41.4% (120/290; 17.7–56.6%) and 6.6% (18/274; 4.1–10.2%), respectively. Multidrug resistance was found in all countries (2.7%; 1.1–4.2%).

Conclusions: Both transcription-mediated amplification (TMA)-based MG assays had a highly superior sensitivity compared to the *mgp*B qPCR. The prevalence of MG and azithromycin resistance was high. Validated and quality-assured molecular tests for MG, routine resistance testing of MG-positive samples and antimicrobial resistance surveillance are crucial. **M. Unemo, Clin Microbiol Infect 2018;24:533**

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https://doi.org/10.1016/j.cmi.2017.09.006

Introduction

Mycoplasma genitalium (MG) frequently causes nongonococcal urethritis (NGU) in male subjects, and is associated with cervicitis, pelvic inflammatory disease, endometritis and probably infertility [1–6].

In many countries worldwide, MG testing is limited or lacking, and increased testing of symptomatic patients in particular is recommended [4,5]. Nucleic acid amplification tests (NAATs) are the only appropriate diagnostic methods. Many commercially available and particularly laboratory-developed diagnostic PCRs have been used [5,7–13]. However, many of these have not been appropriately validated, and none of the companies providing US Food and Drug Administration–approved, automated NAATs for detection of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) have yet obtained Food and Drug Administration approval for an MG NAAT. Recently the new Conformité Européene (CE)/*in vitro* diagnostics (IVD)-marked Aptima *Mycoplasma genitalium* assay (CE/IVD AMG), targeting 16S rRNA, was commercialized for the Panther system (Hologic, San Diego, CA, USA). An earlier non-CE/IVD version of this assay showed promising results in a smaller study which examined only urine specimens [14].

The first-line empiric treatment for male NGU in the 2016 European NGU guideline is doxycycline 100 mg twice daily or 200 mg once daily orally for 7 days. Second-line therapy is azithromycin 500 mg in a single dose on day 1 followed by 250 mg daily for 4 days, or azithromycin 1 g in a single dose orally [3]. In the 2016 European MG guideline, the 5-day azithromycin regimen is the first-line treatment in the absence of macrolide resistance or when the infectious organism's resistance status is unknown. Second-line treatment is moxifloxacin 400 mg once daily for 7 to 10 days [4,5]. During the recent decade, the azithromycin efficacy for treatment of MG infections has declined [4,5,15–17]. This macrolide resistance is primarily caused by mutations at position A2058 or A2059 (*Escherichia coli* numbering) in the 23S rRNA gene [18]. During the most recent years, a declining moxifloxacin efficacy has been documented, particularly in Japan and Australia [4,5,16,17,19–24]. However, surveillance data regarding MG moxifloxacin resistance remain scarce internationally. Moxifloxacin resistance is associated with mutations in the quinolone resistance–determining region (QRDR) of the *par*C gene, primarily at positions S83 and D87 (MG numbering), whereas mutations in *gyr*A presumably play a minor role [23,24].

We performed what is to our knowledge the first large-scale evaluation of the clinical and analytic sensitivity and specificity of the new CE/IVD AMG on the Panther system (Hologic), and described the prevalence of MG, CT, NG and MG resistance–associated mutations for azithromycin and moxifloxacin in unselected patients attending sexually transmitted disease (STD) clinics in Denmark, Norway and Sweden in 2016. For reference testing, a research-use-only MG transcription-mediated amplification (TMA) assay (MG Alt TMA-1 [25]; Hologic), targeting a different 16S rRNA sequence, and a well-recognized laboratory-developed TaqMan real-time *mgp*B qPCR assay, with a sensitivity of five genome equivalents (geq)/reaction [11,26], were used. This was the Nordic Aptima MG Evaluation (NAME) study.

Methods

Patients and biologic specimens

Consecutive male and female subjects attending the STD Clinic at Örebro University Hospital, Örebro, Sweden, from February to December 2016; Olafia Clinic, Oslo University Hospital, Norway, from February 2016 to February 2017; and Bispebjerg University Hospital, Copenhagen, Denmark, from March to June 2016 were enrolled into the study after providing informed consent. Individuals attended the clinics because they had symptoms, because they had had unprotected sex and/or because they had received partner notification. In Sweden and Norway, first-void urine (FVU) was collected from male subjects and two vaginal swabs were simultaneously collected from female subjects. In Denmark, samples included urethral, rectal and/or pharyngeal swabs from male subjects and vaginal, cervical, urethral and/or rectal and pharyngeal swabs from female subjects according to the clinical indication. Two millilitres and 8 to 10 mL of the FVU specimens were promptly transferred to an Aptima Urine Specimen Transport Tube (Hologic) and an AssayAssure Genelock Urine Collection Tube (0.8 mL; Sierra Molecular), respectively. Of the two simultaneously collected vaginal swabs (or the additional specimen types), one was promptly transferred into an Aptima Vaginal Swab Specimen Collection tube (Hologic) and the other into Copan UTM medium (2 mL; Copan). All NAATs were performed in a manner blinded to the results of the other NAATs and generally $\leq$1 week after specimen collection.

External quality assessment (EQA) panel

An EQUALIS (http://www.equalis.se/en/start/) MG EQA panel (2016:01; http://www.equalis.se/sv/vaar-verksamhet/extern-kvalitetssaekring/kvalitetssaekringsprogram/m-r/mycoplasma-genitalium-nukleinsyra-288/) was also tested. The scheme consisted of two MG-negative samples and four MG-positive samples, i.e. one MG-positive urine specimen diluted 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in MG-negative urine.

Analytical inclusivity and specificity panel

Twenty-five unique genome-sequenced MG isolates (unpublished data) and 21 strains of 14 other mycoplasma species (human or phylogenetically closely related) were tested in a blinded manner with the CE/IVD AMG and MG Alt TMA-1. The non-MG mycoplasma strains included five *M. pneumoniae* (Mac, M129, M6696, M2060, M6844), three *M. amphoriforme* (M5572, M6123, A62), two *M. pirum* (NCTC 11702, AMRC C-1555 Zeus 8/11 16) and one each of *M. alvi* (Isley), *M. arginini* (G230), *M. buccale* (CH20247), *M. fermentans*, *M. gallisepticum* (15302), *M. hominis* (PG21), *M. lipophilum* (Maby), *M. orale* (Patt), *M. penetrans* (GTU-54), *M. primatum* (Navel) and *M. salivarium* (PG20). Mycoplasmas were cultured as previously described [27].

CE/IVD AMG assay

The CE/IVD AMG was performed on a Panther system according to the manufacturer's instructions (Hologic).

Reference testing

The MG Alt TMA-1 was performed on a Panther system according to the manufacturer's instructions (Hologic).

The *mgp*B qPCR and DNA extraction using Chelex 100 slurry (Bio-Rad) was performed as previously described [11]. The DNA extracts were tested the same day or were stored at −20°C until use.

Chlamydia trachomatis and Neisseria gonorrhoeae testing

The Aptima Combo 2 (CT/NG) assay was performed on a Panther system according to the manufacturer's instructions (Hologic).

Interpretation of truly positive results

Specimens were considered truly positive if they were positive in a minimum two of the three MG NAATs. Repeated testing of specimens with discrepant results was performed.

Detection of resistance-associated mutations

Resistance-associated mutations in the 23S rRNA and *par*C genes were identified using pyrosequencing and conventional Sanger sequencing, respectively, as described previously [18,23].

Statistical analysis

Fisher's exact test was computed by IBM SPSS Statistics software, with significance set at $p < 0.05$.

Ethics

The study was approved by relevant departmental ethics committees (Regional Committees for Medical and Health Research Ethics South-East 2016/50, Region Hovedstaden H-15018251).

Results

Patients and Mycoplasma genitalium–positive samples

In total, 5269 patients were included. Of these, 1273 (507 female and 766 male subjects), 2547 (721 female and 1826 male subjects) and 1449 (535 female and 914 male subjects) were from Denmark, Norway and Sweden, respectively. The median (range) age of the male subjects was 30 (15–79) years and of the female subjects 27 was (16–65) years, with median ages of 29, 28 and 30 years in Denmark, Norway and Sweden, respectively.

The number of MG-infected patients in Denmark, Norway and Sweden was 115 (9.0%), 125 (4.9%) and 142 (9.8%), respectively. Among the total 382 (7.2%) infected patients, the MG prevalence in male (female) subjects was 8.9% (9.3%), 4.5% (6.0%) and 9.1% (11.0%) in Denmark, Norway and Sweden, respectively. The CT prevalence in the male (female) subjects was 10.7% (10.8%), 8.8% (7.5%) and 6.1% (4.1%) in Denmark, Norway and Sweden, respectively, and the NG prevalence was 8.3% (3.9%), 0.44% (0.14%) and 0.55% (0.93%), respectively. In total, 12.4% of MG-positive male subjects were coinfected with CT (9.9%), NG (1.7%) or CT + NG (0.8%) and 11.4% of MG-positive female subjects were coinfected with CT (8.7%), NG (2.0%) or CT + NG (0.7%).

Clinical sensitivity and specificity

The sensitivity and specificity of the CE/IVD AMG when examining vaginal swabs ($n = 1683$) were 100% and 99.81%, respectively, and 95.65% and 99.61%, respectively, for cervical swabs ($n = 280$). For male subjects, the sensitivity and specificity was 100% and 99.96%, respectively, when examining FVU specimens ($n = 2740$). For male urethral swabs ($n = 754$), the sensitivity and specificity were 96.43% and 99.43%, respectively (Table 1).

The overall sensitivity, specificity, positive predictive value and negative predictive value of the CE/IVD AMG were 99.74% (range in countries, 99.13–100%), 99.84% (99.57–99.96%), 97.94% (95.80–99.21%) and 99.98% (99.91–100%), respectively. The overall sensitivity, specificity, positive predictive value and negative predictive value of the MG Alt TMA-1 were 99.74% (99.13–100%), 100%,

Table 1
Sensitivities, specificities, PPVs and NPVs of different assays in different specimens [11]

Gender (no. of patients)	Specimen (no. of truly positive findings/no. of samples)	Assay[a]	No. of positive results (no. of truly positive results)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Female (1763)	Vaginal (138/1683)	CE/IVD AMG[a]	141 (138)	100	99.81	97.87	99.94
		MG Alt TMA-1[a]	136 (136)	98.55	100	100	99.87
		*mgp*B qPCR[b]	109 (108)	78.26	99.94	99.08	98.09
	Cervical (23/280)	CE/IVD AMG[a]	23 (22)	95.65	99.61	95.65	99.61
		MG Alt TMA-1[a]	21 (21)	91.30	100	100	99.23
		*mgp*B qPCR[b]	18 (18)	78.26	100	100	98.09
	Urethral (26/290)	CE/IVD AMG[a]	26 (25)	96.15	99.62	96.15	99.62
		MG Alt TMA-1[a]	25 (25)	96.15	100	100	99.62
		*mgp*B qPCR[b]	13 (13)	50.00	100	100	95.31
	Rectal (3/37)	CE/IVD AMG [a]	4 (3)	100	97.06	75.00	100
		MG Alt TMA-1[a]	3 (3)	100	100	100	100
		*mgp*B qPCR[b]	1 (1)	33.33	100	100	94.44
	Pharyngeal (0/74)	CE/IVD AMG [a]	0	NA	NA	NA	NA
		MG Alt TMA-1[a]	0	NA	NA	NA	NA
		*mgp*B qPCR[b]	0	NA	NA	NA	NA
Male (3506)	FVU (165/2740)	CE/IVD AMG[c]	166 (165)	100	99.96	99.40	100
		MG Alt TMA-1[c]	165 (165)	100	100	100	100
		*mgp*B qPCR[d]	129 (127)	76.97	99.88	97.69	98.54
	Urethral (56/754)	CE/IVD AMG[a]	58 (54)	96.43	99.43	93.75	99.71
		MG Alt TMA-1[a]	54 (54)	96.43	100	100	99.71
		*mgp*B qPCR[b]	38 (38)	67.86	100	100	97.49
	Rectal (15/237)	CE/IVD AMG [a]	16 (15)	100	99.55	93.75	100
		MG Alt TMA-1[a]	15 (15)	100	100	100	100
		*mgp*B qPCR[b]	8 (8)	53.33	100	100	96.94
	Pharyngeal (4/260)	CE/IVD AMG [a]	4 (4)	100	100	100	100
		MG Alt TMA-1[a]	4 (4)	100	100	100	100
		*mgp*B qPCR[b]	1 (1)	25.00	100	100	98.84

AMG, Aptima *Mycoplasma genitalium* assay; CE, Conformité Européene; FVU, first-void urine; IVD, *in vitro* diagnostics; NA, not applicable; NPV, negative predictive value; PPV, positive predictive value; qPCR, quantitative real-time PCR.

[a] Total of 13% of primary swab sample (400 μL taken from 3 mL Aptima transport medium) analysed in assay after nucleic acid extraction.

[b] Total of 0.5% of primary swab sample analysed in assay after nucleic acid extraction.

[c] Total of 200 μL of primary FVU sample analysed in assay after nucleic acid extraction.

[d] Total of 35 μL of primary FVU sample analysed in assay after nucleic acid extraction.

Table 2
Sensitivities, specificities, PPVs and NPVs based on patient infection status [11]

Assay	Population (no. of infected patients/no. of included patients)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CE/IVD AMG	Denmark (115/1273)	99.13 (114/115)	99.57 (1153/1158)	95.80 (114/119)	99.91 (1153/1154)
	Norway (125/2547)	100 (125/125)	99.96 (2421/2422)	99.21 (125/126)	100 (2421/2421)
	Sweden (142/1449)	100 (142/142)	99.85 (1305/1307)	98.61 (142/144)	100 (1305/1305)
	Total (n = 5269)	99.74 (381/382)	99.84 (4879/4887)	97.94 (381/389)	99.98 (4879/4880)
MG Alt TMA-1	Denmark (115/1273)	99.13 (114/115)	100 (1158/1158)	100 (114/114)	99.91 (1158/1159)
	Norway (125/2547)	100 (125/125)	100 (2422/2422)	100 (125/125)	100 (2422/2422)
	Sweden (142/1449)	100 (142/142)	100 (1307/1307)	100 (142/142)	100 (1307/1307)
	Total (n = 5269)	99.74 (381/382)	100 (4887/4887)	100 (381/381)	99.98 (4887/4888)
*mgp*B qPCR	Denmark (115/1273)	73.91 (85/115)	100 (1158/1158)	100 (85/85)	97.47 (1158/1188)
	Norway (125/2547)	81.60 (102/125)	100 (2422/2422)	100 (102/102)	99.06 (2422/2445)
	Sweden (142/1449)	73.24 (104/142)	99.69 (1303/1307)	96.30 (104/108)	97.17 (1303/1341)
	Total (n = 5269)	76.18 (291/382)	99.92 (4883/4887)	98.64 (291/295)	98.17 (4883/4974)

AMG, Aptima *Mycoplasma genitalium;* CE, Conformité Européene; FVU, first-void urine; IVD, *in vitro* diagnostics; NA, not applicable; NPV, negative predictive value; PPV, positive predictive value; qPCR, quantitative real-time PCR.

100% and 99.98% (99.91–100%), and for the *mgp*B qPCR were 76.18% (73.24–81.60%), 99.92% (99.69–100%), 98.64% (96.30–100%) and 98.17% (97.17–99.06%), respectively (Table 2).

Both the CE/IVD AMG and MG Alt TMA-1 showed correct results for all six samples in the EQUALIS MG EQA panel (2016:01). The *mgp*B qPCR missed the two samples with lowest MG concentration. For comparison, the results for this EQA panel distributed to 29 Scandinavian laboratories is provided in Fig. 1.

Analytical inclusivity and specificity panel

All 25 MG strains were detected at <10 geq per reaction, and several strains were detected in concentrations below the limit of detection for the *mgp*B PCR assay. All strains of *M. amphoriforme* (n = 3), *M. pirum* (n = 2) and *M. alvi* (n = 1) were reproducibly positive in the CE/IVD AMG down to concentrations <50 geq per reaction. No cross-reaction was observed in the MG Alt TMA-1.

Macrolide resistance–associated mutations

Macrolide resistance–associated 23S rRNA gene mutations were detected in 41.4% of samples (39.3% (68/173) and 44.4% (52/117) in male and female samples, respectively) (Table 3).

The 23S rRNA gene mutation A2059G was predominant in Denmark (53.5% of mutated samples) and Norway (59.6%), while A2058G (50.0%) was slightly more common in Sweden. The A2059G and A2058G mutations accounted for 96.7% of mutated samples (Table 3).

Moxifloxacin resistance–associated mutations

The rate of possible fluoroquinolone resistance–associated *par*C QRDR mutations was 6.6% (88.9% of samples from male subjects) (Table 4).

The most frequent *par*C QRDR amino acid alterations were D87N (n = 5) and S83N (n = 5), followed by S83I (n = 4) and D87H (n = 3). These *par*C alterations accounted for 94.4% of mutated samples (Table 4).

Multidrug resistance

In Denmark, Sweden and Norway, three (4.2%), three (3.1%) and one (1.1%) sample, respectively, comprised both macrolide and possible fluoroquinolone resistance–associated mutations (Table 5).

Discussion

In what is to our knowledge the first large-scale prospective evaluation of a novel test (including 5269 consecutive

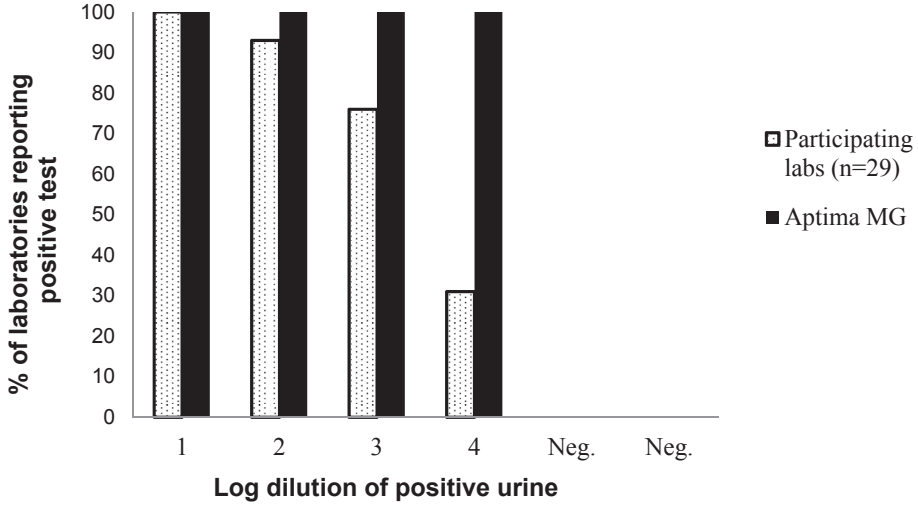

Fig. 1. Results of 29 Scandinavian diagnostic laboratories and CE/IVD Aptima *Mycoplasma genitalium* assay (CE/IVD AMG) and MG Alt TMA-1 assay (this study; collectively referred as Aptima MG because both assays detected 100% of positive samples) when testing EQUALIS *Mycoplasma genitalium* external quality assessment panel (2016:01; http://www.equalis.se/en). CE, Conformité Européene; IVD, *in vitro* diagnostics.

Table 3
Prevalence of macrolide resistance–associated 23S rRNA gene mutations in *Mycoplasma genitalium*–positive samples in Denmark, Norway and Sweden

Mutation[a]	Frequency[b]			
	Denmark	Norway	Sweden	Total
A2059G	30.3 (23/76)	33.7 (34/101)	8.0 (9/113)	22.8 (66/290)
A2058G	26.3 (20/76)	19.8 (20/101)	8.8 (10/113)	17.2 (50/290)
A2058T	—	2.0 (2/101)	0.9 (1/113)	1.0 (3/290)
A2059C	—	1.0 (1/101)	—	0.3 (1/290)
Wild type	43.4 (33/76)	43.6 (44/101)	82.3 (93/113)	58.6 (170/290)
Nontypeable	9	24	29	62
Total mutated	56.6 (43/76)	56.4 (57/101)	17.7 (20/113)	41.4 (120/290)

[a] Nucleotide positions in 23S rRNA gene are given according to *Escherichia coli* numbering.
[b] Frequency data are presented as % (no. of samples with mutations/no. of successfully sequenced samples). Only one sample per positive patient included.

Table 4
Prevalence of possible fluoroquinolone resistance–associated *par*C mutations (mutations in QRDR of *par*C) in *Mycoplasma genitalium*–positive samples in Denmark, Norway and Sweden

Mutation[a]	Frequency[b]			
	Denmark	Norway	Sweden	Total
D87N[c]	—	2.0 (2/98)	3.1 (3/98)	1.8 (5/274)
S83N[d]	1.3 (1/78)	—	4.1 (4/98)	1.8 (5/274)
S83I[c]	2.6 (2/78)	1.0 (1/98)	1.0 (1/98)	1.5 (4/274)
D87H	—	1.0 (1/98)	2.0 (2/98)	1.1 (3/274)
S83R[c]	1.3 (1/78)	—	—	0.4 (1/274)
WT, silent or outside QRDR	95 (74/78)	96 (94/98)	90 (88/98)	93 (256/274)
Nontypeable	10	27	44	81
Total mutated	5.1 (4/78)	4.1 (4/98)	10.2 (10/98)	6.6 (18/274)

MIC, minimum inhibitory concentration; QRDR, quinolone resistance–determining region (estimated at *par*C amino acid codons 80–90); WT, wild type.
[a] Amino acid positions in *par*C are given according to *M. genitalium* numbering.
[b] Frequency data are presented as % (no. of samples with mutations/no. of successfully sequenced samples). Only one sample per positive patient included.
[c] Mutations where *in vitro* MIC determination has demonstrated significantly elevated MICs of moxifloxacin.
[d] One strain evaluated had moxifloxacin-susceptible phenotype (Jensen et al., unpublished data).

Table 5
Prevalence of *Mycoplasma genitalium*–positive samples with possible multidrug resistance (both 23S rRNA gene and *par*C resistance–associated mutations) in Denmark, Norway and Sweden

Mutation	Frequency[a]			
	Denmark	Norway	Sweden	Total
MRAM and D87N		1.1 (1/92)	2.1 (2/97)	1.2 (3/260)
MRAM and S83N	1.4 (1/74)		1.0 (1/97)	0.8 (2/260)
MRAM and S83R	1.4 (1/74)			0.4 (1/260)
MRAM and S83I	1.4 (1/74)			0.4 (1/260)
MRAM and WT, silent or outside QRDR	52.7 (39/74)	54.3 (50/92)	17.5 (17/97)	106/263
Total mutated	4.2 (3/71)	1.1 (1/92)	3.1 (3/97)	2.7 (7/260)

MRAM, macrolide resistance–associated mutation; QRDR, quinolone resistance–determining region (estimated at *par*C amino acid codons 80–90); WT, wild type.
[a] Frequency data are presented as % (no. of samples with mutations/no. of successfully sequenced samples). Only one sample per positive patient included.

symptomatic and asymptomatic patients in the three Scandinavian countries), the new CE/IVD AMG showed a high clinical and analytical sensitivity and specificity when used to examine urogenital and extragenital specimens. The clinical sensitivity (99.74%) was significantly higher compared to the *mgp*B qPCR (76.18%). MG infections frequently have low bacterial loads, i.e. 100 times lower compared to CT infections [28], and for these infections, the Aptima MG assays, using larger volume of the primary sample (Table 1), target capture of 16S rRNA (up to 100–1000 copies per cell) and TMA, likely have a superior clinical sensitivity compared to any PCRs targeting single- or multiple-copy genes.

The sensitivity examining recommended vaginal swabs [4] was higher (100%), with high specificity (99.81%), compared to invasive cervical (95.65%) and urethral swabs (96.15%), which is consistent with previous studies [4,5,29]. For male subjects, the sensitivity examining recommended FVU specimens [4] was also higher (100%) compared to invasive urethral swabs (96.43%). However, these differences were not significant as a result of the small number of samples. The sensitivity examining rectal swabs was 100% in both genders.

The clinical specificities of the CE/IVD AMG (99.84%) and MG Alt TMA-1 (100%) were also high. For the first time, the analytical specificity of these MG assays was comprehensively investigated as well. Surprisingly, all strains of *M. amphoriforme, M. pirum* and *M. alvi* were detected in low concentrations in the CE/IVD AMG, while the MG Alt TMA-1 showed 100% analytical specificity. The knowledge regarding these mycoplasma species and their prevalence in human specimens is limited. *M. alvi* has been found mainly in the bovine gastrointestinal tract, and this cross-reaction appears clinically irrelevant [30]. *M. pirum* is considered an HIV-associated mycoplasma species [31] that has been found in rectal specimens from men who have sex with men [32]. Four male urethral swabs (0.5%) were positive only in the CE/IVD AMG, but this finding might only reflect a lower limit of detection of this assay. Nevertheless, particularly for HIV-positive men who have sex with men, a positive CE/IVD AMG rectal test might have to be treated with caution. *M. amphoriforme* has been found in patients with primary antibody deficiency [33] and respiratory infections [34], but evidence of disease associations is lacking. None of the pharyngeal swabs ($n = 334$) tested positive only in the CE/IVD AMG. Consequently, based on this as well as the overall clinical specificity of 99.84% of the CE/IVD AMG, the clinical relevance of possible cross-reactivity with these three mycoplasma species appears to be minimal.

The prevalence of MG was significantly higher in Denmark (9.0%) and Sweden (9.8%) compared to Norway (4.9%) ($p < 0.05$). At the Olafia Clinic, Norway, comprehensive MG testing, treatment and contact notification for both symptomatic and asymptomatic individuals have been performed since 2005 (as for CT), and consequently MG prevalence might have declined. Overall, MG was very common, which might be because MG is, at least in Denmark and Sweden, not routinely tested for in all attendees of STD clinics.

Macrolide resistance was significantly less prevalent in Sweden (18%) compared to Norway (56%) and Denmark (57%) ($p < 0.05$). This likely reflects that azithromycin 1 g, which effectively induces or selects for macrolide resistance, has been used as first-line treatment for NGU and CT infections in Denmark and Norway for many years. In Sweden, doxycycline has always been the recommended first-line treatment for these infections. On the basis of the high resistance levels to macrolides in Scandinavia and internationally [4,5,15–17], and the frequent selection of resistance during azithromycin treatment, a test of cure after >3 weeks is crucial even in patients infected with macrolide-susceptible MG strains [4]. Furthermore, ideally, macrolide resistance testing should be performed on all MG-positive samples before treatment is initiated [4,5]. The level of potential moxifloxacin resistance–associated mutations was relatively low in Scandinavia, but it is increasing. It was higher in Sweden (10.2%) compared to Denmark (5.1%) and Norway (4.1%). However, the *par*C S83N alteration accounted for 40% of the mutations in Sweden, and this alteration might not significantly increase the

moxifloxacin minimum inhibitory concentrations (MIC; 0.125 mg/L for one isolate; Jensen et al., unpublished data). Moxifloxacin resistance–associated mutations were mainly detected in male subjects (88.9%). This might indicate that these male subjects belong to a high-frequency transmitting population and have repeatedly been treated with moxifloxacin for MG infection or persistent NGU suspected to be due to MG. This hypothesis needs further detailed testing. Studies in Japan and Australia have shown high prevalences of *par*C QRDR mutations, with treatment failure of moxifloxacin [4,5,16,17,20–22,24]. In our study, potentially multidrug-resistant MG was found in all countries (2.7%; 1.1–4.2%). Multidrug resistance in MG is a major concern. Pristinamycin is the only third-line treatment available [4], but it is not 100% effective [21].

Major strengths of our study include using a large sample size (5269 patients); testing both urogenital and extragenital specimens from consecutive symptomatic and asymptomatic individuals in three countries (demographic and geographic diversity) which effectively assess the NAATs and provide reliable MG, CT and NG prevalence figures; using three different NAATs on all specimens to detect truly infected patients; and sequencing for both macrolide and fluoroquinolone resistance. Limitations include that details regarding the patients' demographics, risk factors, and presenting clinical signs and symptoms were unavailable.

In conclusion, the new CE/IVD AMG assay performed in the fully automated Panther system showed a high clinical sensitivity and specificity, and it offers a simple, rapid, sensitive and specific MG diagnostics which can easily be combined with detection of CT, NG and *Trichomonas vaginalis*. The *mgp*B reference PCR missed >20% of positive samples, which emphasizes the fact that earlier published MG prevalence figures internationally are underestimated and many patients may have received false-negative results. However, as for all commercially available NAATs, the cost per test of the CE/IVD AMG assay is significantly higher than laboratory-developed diagnostic PCRs, e.g. the *mgp*B PCR used in this study, and in general it is crucial to ensure that appropriate NAATs are also affordable in less-resourced settings. Routine resistance testing of all MG positive samples, at least for macrolide resistance–associated 23S rRNA gene mutations, test of cure after >3 weeks, and regular national and international surveillance of MG resistance are all imperative. Furthermore, use of doxycycline instead of azithromycin for empirical first-line therapy of NGU is recommended [3,5]. The importance of several *parC* mutations as well as the possible impact of *gyr*A mutations on moxifloxacin MICs remain largely unknown, and culturing samples with MG strains carrying mutations in order to associate to phenotypic resistance should be prioritized. Finally, novel antimicrobials for MG treatment are essential. For example, lefamulin, gepotidacin and zoliflodacin deserve further attention, and dual antimicrobial therapy needs to be considered [5].

Transparency Declaration

Hologic provided the CE/IVD AMG and financial support for the testing; however, Hologic had no role in data collection or interpretation. The study was also supported by the Örebro County Council Research Committee and the Foundation for Medical Research at Örebro University Hospital, Örebro, Sweden. All authors report no conflicts of interest relevant to this article.

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