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ORIGINAL ARTICLE

Clinical evaluation of commercial PCR assays for antimicrobal resistance in *Mycoplasma genitalium* and estimation of resistance-mediated mutation prevalence in Moscow and Moscow region

Elizaveta Dmitrievna Shedko[1] · Guzel Anvarovna Khayrullina[1] · Elena Nikolaevna Goloveshkina[1] · Vasiliy Gennadevich Akimkin[1]

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Abstract

Mycoplasma genitalium is a widespread sexually transmitted infection (STI) with growing rate of antimicrobials resistance. In our study, 137 vaginal and 131 urethral *M. genitalium*–positive swabs were sequentially collected through the work of Reference Center for STI during 2019. For prevalence evaluation of macrolide-resistance mutations three commercially available kits were used: AmpliSens® *M. genitalium*-ML/FQ-Resist-FL (Central Research Institute of Epidemiology, Russia), ResistancePlus® MG (SpeeDx, Australia), and S-DiaMGRes™ (Diagenode, Belgium). Macrolide resistance mutations were detected in 16% (43 of 268) of samples. Diagnostic characteristics were evaluated against Sanger sequencing. For AmpliSens® *M. genitalium*-ML/FQ-Resist-FL specificity was shown to be 100% (CI 95%, 98.4–100), and sensitivity was 90.7% (CI 95%, 77.9–97.4). ResistancePlus® MG specificity was 100% (CI 95%, 98.3–100), and sensitivity was 92.1% (CI 95%, 78.6–98.3). S-DiaMGRes™ specificity was shown to be 88.6% (CI 95%, 83.9–92.4), and sensitivity was 100% (CI 95%, 84.4–100). Mutations of *parC* gene region were detected in 14.5% (38 of 268) using AmpliSens® *M. genitalium*-ML/FQ-Resist-FL with further validation by Sanger sequencing. Of studied samples, 6.3% (17 of 268) contained both antimicrobials of class resistance mutations. Prevalence of macrolide-resistant *M. genitalium* in Moscow was 21.7% (23 of 106) and of fluoroquinolone-resistant *M. genitaliuim* was 20.8% (22 of 106). In Moscow region, macrolide-resistant *M. genitalium* were 12.3% (20 of 162) and 9.9% (16 of 162) of fluoroquinolone-resistant *M. genitalium*. All three kits can be used both for epidemiological monitoring of *M. genitalium* presence and mutation prevalence estimation. In Moscow, macrolide- and fluoroquinolone-resistant mutant prevalence increased in 3.9 and 2.7 times in 3 years.

Keywords *Mycoplasma genitalium* · Antibiotic resistance gene determinants · Molecular diagnostics · QRDR · 23S rRNA · PCR · Macrolide · Fluoroquinolone

Introduction

Mycoplasma genitalium is the facultative anaerobic smallest known microorganism with genome consisting of approximately 580-kb pairs [1]. Firstly discovered in 1981 [2], now *M. genitalium* is a cause of widespread STI with prevalence ranging from 0.7 to 3.3% in general population, 0.9% in pregnant women, 3.2% in men who have sex with men (MSM), and 15.9% in female sex workers [3]. *Mycoplasma genitalium* infection can lead to urethritis in males and cervicitis, endometritis, pelvic inflammatory disease, possibly preterm birth, tubal factor infertility, and ectopic pregnancy in females [4]. It has been shown that *M. genitalium* may be linked to increased risk of HIV infection [5].

Antimicrobial resistance is a worldwide rapidly emerging problem [6]. It is extremely difficult to culture *M. genitalium* which strictly limits culture-based antimicrobial tests [7]. Macrolides and fluoroquinolones are the most commonly

✉ Elizaveta Dmitrievna Shedko
shedko@cmd.su

[1] Federal Budget Institution of Science "Central Research Institute of Epidemiology" of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance, Moscow, Russia

used antimicrobials in treatment of *M. genitalium* infections. According to European guidelines on *Mycoplasma genitalium* infection [8], only azithromycin, josamycin, moxifloxacin pristinamycin, and doxycycline are the antimicrobials of choice, and in accordance with the BASHH UK national guidelines, moxifloxacin, doxycycline, pristinamycin, and minocycline are used [9].

It is not known for *M. genitalium* to carry any extrachromosomal DNA [10]; therefore, all antimicrobial resistances are determined by chromosomal mutations. Macrolides interact with A2058 (*Escherichia coli* numeration) position of 23S rRNA by formation of hydrogen bond [11]. Macrolide resistance in *M. genitalium* is determined by mutations in 23S rRNA gene V region [12]. The most common mutations are A2071 and A2072 (A2058 and A2059 *E. coli* numeration) [13]. It was also shown that mutations A752, C2038, and T2185 (*E. coli* numeration) may lead to macrolide resistance [14]; furthermore, it was shown previously that A2062 position leads to failure in josamycin treatment [15]. Fluoroquinolones inhibit DNA gyrase and topoisomerase IV encoded by *gyrA* and *parC* genes, respectively, which are essential for bacterial DNA synthesis [16]. It was previously shown that G205A/Ala(69)→Thr, A248T/Ser(83)→Ile, and G259T/Asp87(84)→Tyr (*E. coli* numeration) mutations in *parC* gene region for *M. genitalium* are connected with fluoroquinolone resistance [17–19].

It was reported that, in Europe, rates of *M. genitalium* macrolide resistance usually exceeding 50% with a trend is still growing ($r^2 = 0.531$; $p = 0.101$) [20]. In European guidelines on *Mycoplasma genitalium* infections, it is strongly recommended to follow every positive *M. genitalium* test with macrolide resistance detecting assay [8], as well as in the BASHH UK national guidelines [9].

AmpliSens® *M. genitalium*–ML/FQ-Resist-FL (Central Research Institute of Epidemiology) kit detects mutations in 23S rRNA gene V region and mutations in *parC* gene. It is a unique method based on oligonucleotide probes complimentary to wild type (WT) of *M. genitalium* which allows detecting maximal spectrum of resistance-associated mutations in abovementioned regions. Detection is carried out simultaneously with no mutation differentiation within one region. ResistancePlus® MG (SpeeDx) kit simultaneously detects A2058G, A2059G, A2058T, A2058C, or A2059C mutations on one channel. S-DiaMGRes™ (Diagenode) simultaneously detects mutations in A2058 and A2059 positions using FAM (Ex./Em.: 494/520 nm) and YY (Ex./Em.: 530/549 nm), respectively, thus resulted obtained allow differentiating position of studied sample. All of the used reagent kits simultaneously detect *M. genitalium* DNA and resistance-associated mutations.

In our study, we evaluated prevalence of urogenital *M. genitalium* macrolide and fluoroquinolone resistance mutations in samples collected in Moscow during 2019 year through the work of Reference Center for STI of Central Research Institute of Epidemiology (Moscow, Russia) and compared the performance commercially available kits: newly developed real-time PCR-based AmpliSens® *M. genitalium*–ML/FQ-Resist-FL (Central Research Institute of Epidemiology), ResistancePlus® MG (SpeeDx), and S-DiaMGRes™ (Diagenode). As a reference method, Sanger sequencing was used.

Materials and methods

Clinical samples

All samples have been sequentially collected from February to June of 2019 year at the Center of Molecular Diagnostics (Central Research Institute of Epidemiology, Russia) through the work of Reference Center for STI. In our study, we included only *M. genitalium*–positive 137 female vaginal and 131 male urethral swabs determined previously with «AmpliSens® *N. gonorrhoeae/C. trachomatis/M. genitalium/T. vaginalis*-MULTIPRIME-FRT» (Central Research Institute of Epidemiology, Russia). Age median of patients was 29 ± 5.64 years. One-hundred six samples were from Moscow, and 162 were from Moscow region. Only data on age and sex of the patients were presented with no data obtained on risk profile or sexual behavior. All collected samples were stored at –70 °C before further processing.

DNA extraction

All samples were extracted using DNA-sorb-AM (AmpliSens, Russia) extraction kit. Briefly, all samples were vortexed and centrifuged for 5 s to remove excess drops on the lid. After, 100 μl of clinical sample was placed in the 1.5-ml Eppendorf tube and proceeded according to manufacturer's instructions. IC included in AmpliSens® *M. genitalium*–ML/FQ-Resist-FL (Central Research Institute of Epidemiology, Russia), ResistancePlus® MG (SpeeDx, Australia), and S-DiaMGRes™ (Diagenode, Belgium) reagents kits were added at the stage of extraction. All samples were eluted in 100 μl.

Amplification

Extractions for each used qPCR kit were performed separately using appropriate IC according to manufacturer's instructions. Both ResistancePlus® MG and S-DiaMGRes™ assays were performed on CFX96® Touch while *M. genitalium*–ML/FQ-Resist-FL (AmpliSens) assay was performed on Rotor-Gene® 6000.

Amplification data analysis

Data obtained using S-DiaMGRes™ (Diagenode) assay was analyzed using CFX Manager v 3.1 according to manufacturer's results interpretation guidelines. For ResistancePlus® MG (SpeeDx) Ugen Tech FastFined v 3.5.8 software was used. For *M. genitalium*–ML/FQ-Resist-FL (AmpliSens) excel macro-AmpliSens® *M. genitalium*–ML/FQ-Resist (RUS) was used.

Sequencing

Sanger sequencing of 23S rRNA gene region and *par*C QRDR was used as a reference standard method to determine mutations of macrolide resistance. Sanger sequencing was performed with previously described primers [12, 21]. Sequencing was performed using 3500 × l Genetic Analyzer (Applied Biosystems, USA). Sequences were analyzed using FinchTV v 1.4.0 software. All studied samples were sequenced.

Statistical analysis

Statistical analysis was performed using Westgard QC (http://tools.westgard.com) and Epitools (https://epitools.ausvet.com.au) websites.

Results

M. genitalium detection

Presence of *M. genitalium* in all of the 268 collected samples was confirmed by real-time PCR amplification of *M. genitalium gyrB* gene region with «AmpliSens® *M. genitalium*–screen-titer-FL» (Central Research Institute of Epidemiology, Russia) qPCR kit after thawing and DNA extraction. ResistancePlus® MG (SpeeDx) detected *M. genitalium* in 263 of 268 samples with positive agreement 98.1% while two other kits detected *M. genitalium* in every tested sample with positive agreement of 100%. In all the samples, appropriate IC was detected by qPCR. There was no difference shown for all three used reagents kits in performance on female vaginal or male urethral swabs.

M. genitalium macrolide resistance mutation detection

It was shown that 16% (43 of 268) samples contained 23S rRNA gene mutations after sequencing assay was performed (Table 1). All chromatograms were analyzed on having double peaks. In case of A2062 mutations, double peaks were always observed which allows us to conclude that all 5 samples had mixed *M. genitalium* strains containing both 23S rRNA gene mutants and wild-type cells.

For macrolide resistance detection only S-DiaMGRes™ had sensitivity of 100%, while AmpliSens® *M. genitalium*–ML/FQ-Resist-FL and ResistancePlus® MG had 92.1% and 90.7%, respectively. Specificities for AmpliSens® *M. genitalium*–ML/FQ-Resist-FL, ResistancePlus® MG, and S-DiaMGRes™ were determined as 100%, 100%, and 88.6%, respectively. Both specificity and sensitivity were determined using Sanger sequencing reference method (Table 2).

M. genitalium fluoroquinolone resistance mutations detection

Mycoplasma genitalium–ML/FQ-Resist-FL (AmpliSens) kit can also determine fluoroquinolone resistance mutations in QRDR of *parC* gene. As a reference method, Sanger sequencing of *parC* gene region containing positions A246, T247, A248, G249, G259, and A260 was used (Table 1). Of all specimens 14.5% (38 of 268) contained QRDR mutation. Sample with T251C mutation was excluded from calculations for it was not described previously as determining fluoroquinolone resistance. Analysis of sequencing chromatographs showed no additional or double peaks. Sensitivity and specificity AmpliSens® *M. genitalium*–ML/FQ-Resist-FL were 100% and 100%, respectively (Table 3).

Prevalence of *M. genitalium* mutants with parC and 23S gene mutations

It is of great importance that 6.3% (17 of 268) of *M. genitalium* specimens contained both macrolide and fluoroquinolone resistance mutations which were determined using AmpliSens® *M. genitalium*–ML/FQ-Resist-FL and verified with reference method.

Prevalence of *M. genitalium* mutants in Moscow and Moscow region

In our study, overall macrolide resistance prevalence was 16% (43 of 268). Prevalence of macrolide-resistant *M. genitalium* in Moscow was shown to be 21.7% (23 of 106) and 12.3% (20 of 162) in Moscow region. Prevalence of macrolide-resistant mutants among female patients was 9.5% (13 of 137), and the mean age of the group was 26.8 ± 5.2 while, among male patients, there were 22.9% (30 of 131) with the mean age 30 ± 4.4.

Overall prevalence of fluoroquinolone-resistant *M. genitalium* was shown to be 14.5% (38 of 268). Prevalence of QRDR *M. genitalium* in Moscow was shown to be 20.8% (22 of 106) and 9.9% (16 of 162) in Moscow region. Among female patients, it was shown to be 14.6% (20

Table 1 *M. gentalium* Sanger sequencing results

	Gene region												
	23S rRNA V					*ParC*							
Mutation	A2059G	A2058G	A2058T	A2062C	A2062G	G259A	G259T	G249T	G249C	G249A	A248C	A260T	T251C
Number of mutats	27	8	3	4	1	16	1	9	1	7	2	1	1
Total	43					38							

of 137), and the mean age of the group was 27.6 ± 5.3, and among male patients was 13.7% (18 of 131) with the mean age 31.9 ± 5.4.

It was also shown that the amount of *M. genitalium* containing both macrolide and fluoroquinolone mutations was 3.7%.

Discussion

Antimicrobial resistance is one of the major concerns, both in sense of health and economics. According to Aslam et al. [22], growing threat of antibiotic resistance by 2050 will lead to death of 444 million people with economic cost of eliminating the problem amounting about $120 trillion. In our study, we evaluated three commercially available assays on detection of 23S rRNA gene mutants *M. genitalium*.

Commercial kits of analytical characteristics

It is important to highlight that we only included *M. genitalium*–positive samples in our study. Thus, sensitivity and specificity on *M. genitalium* detection could not be estimated. We only evaluated positive agreement with «AmpliSens® *M. genitalium*–screen-titer-FL» and further Sanger sequencing.

Diagnostic and analytical characteristics of both S-DiaMGRes™ and ResistancePlus® MG assays were previously evaluated in other studies. It is important to note that, in present study, no *M. genitalium*–negative samples were used. For ResistancePlus® MG, specificity was evaluated in our study as 100% (CI 95% 98.3−100) which is accordant to previous studies, and sensitivity was evaluated as 92.1% which is lower than sensitivity in other studies; however, this value is in the boundaries of CI 95% measured previously [23, 24]. For S-DiaMGRes™, specificity was 88.5% that differs from another study [25], while it has the highest sensitivity of 100%.

Discordant results

Of all the commercial kits, only S-DiaMGRes™ (Diagenode) allows to determine not only that *M. genitalium* has 23S mutations but also whether it is in A2058 or A2059 positions. In our study, using S-DiaMGRes™ 76.9% (30 of 39) 23S rRNA gene mutants were determined in accordance with Sanger sequencing. Among the discordant results, 63% (17 of 27) were false-positive results on WT samples, and 37% (10 of 27) were the discordant result on the type of mutation. Authors suggest that, even if correct type of mutation detection is important for research purposes, it is not of great importance for clinical use because both A2058 and A2059 mutations cause

Table 2 *M. gentalium* macrolide mutation detection in selected positive *M. genitalium* samples

Kit	TP[1]	FP[2]	FN[3]	TN[4]	Specificity, %	CI[5] 95%	Sensitivity, %	CI[5] 95%
AmpliSens® *M. genitalium*–ML/FQ-Resist-FL	*39*	0	4	*225*	100	98.4−100	90.7	77.9–97.5
ResistancePlus® MG	*35*	3	0 (5*)	*225*	100	98.3−100	92.1	78.6–98.3
S-DiaMGRes™	*30*	27 (17 + 10**)	0	*211*	88.6	83.9−92.4	100	88.4−100

[1] TP, true positive

[2] FP, false positive

[3] FN, false negative

[4] TN, true negative

[5] CI, confidence interval

*Unidentified *M. genitalium* samples were not included in calculations of diagnostic characteristics for mutation detection was evaluated not overall kit performance

**17 of 27 were false-positive results on WT samples, and 10 of 27 were discordant result on the type of mutation

Table 3 *Mycoplasma gentalium* fluoroquinolone mutation detection in selected positive *M. genitalium* samples

Kit	TP[1]	FP[2]	FN[3]	TN[4]	Specificity, %	CI[5] 95%	Sensitivity, %	CI[5] 95%
AmpliSens® *M. genitalium*–ML/FQ-Resist-FL	*38*	0	0	*229*	100	98.4–100	100	90.8–100

[1] TP, true positive

[2] FP, false positive

[3] FN, false negative

[4] TN, true negative

[5] CI, confidence interval

macrolide resistance. For *M. genitalium*–ML/FQ-Resist-FL (AmpliSens), the only discordant results obtained were in samples containing both WT and 23S A2062C mutants. Authors imply that having a mixed mutant A2062C and WT sample may have an impact on mutant detection for both types of amplicons are synthetized during multiplex qPCR, but further research on that is required. The only discordant results for ResistancePlus® MG (SpeeDx) were false-negative results on *M. genitalium* DNA detection in samples.

Prevalence of *M. genitalium* mutants in Moscow and Moscow region

Two studies presented on antimicrobial-resistant *M. genitalium* mutant rate evaluation in Moscow and Moscow region. According to study performed on samples from dermatovenereological clinic in Moscow from 2014 to 2018, increment of macrolide-resistant mutants was 6.0% [13]. It was previously shown by Shipitsyna et al. [26] that prevalence of macrolide-resistant mutants in Moscow was 5.6% in samples collected 2013–2016.

Overall prevalence of fluoroquinolone-resistant *M. genitalium* was shown to be 14.5% (38 of 268). Prevalence of QRDR *M. genitalium* in Moscow was shown to be 20.8% (22 of 106) and 9.9% (16 of 162) in Moscow region. Among female patients, it was shown to be 14.6% (20 of 137), and the mean age of the group was 27.6 ± 5.3, and among male patients was 13.7% (18 of 131) with the mean age 31.9 ± 5.4. Samples obtained from dermatovenereological patients in Moscow from 2014 to 2018 had 7.1% fluoroquinolone-resistant *M. genitalium* mutants [13], and in Shipitsyna et al. study [26], prevalence of fluoroquinolone-resistant *M. genitalium* in Moscow was 7.6%.

Shipitsyna et al. [26] study was also performed using samples obtained from the Center of Molecular Diagnostics of Central Research Institute of Epidemiology in Moscow. Thus, authors imply that prevalence of antimicrobials resistance in Moscow increased at an alarming rate with macrolide resistance increasing in 3.9 times and of fluoroquinolone resistance—in 2.7 times in 3 years.

It was also shown that the amounts of *M. genitalium* containing both macrolide and fluoroquinolone mutations were dramatically increased in Moscow from 1.3% [26] to 3.7%, in 2.7 times.

Also, it is of interest that one of the QRDR mutants contained mutation T251C that was not described previously. We excluded it from statistical analysis for data on phenotypical susceptibility of fluoroquinolones was not presented. Further studies on the matter are required.

Conclusion

In our study, we evaluated the performance of three commercially available assays on macrolide resistance. AmpliSens® *M. genitalium*–ML/FQ-Resist-FL (Central Research Institute of Epidemiology, Russia), ResistancePlus® MG (SpeeDx, Australia), and S-DiaMGRes™ (Diagenode, Belgium) are able to determine 23S rRNA gene mutations. Thus, all of them can be used both for epidemiological monitoring of *M. genitalium* and mutation prevalence estimation. It was shown that prevalences of *M. genitalium* containing macrolide, fluoroquinolone, and both mutations in Moscow in 3 years from 2016 to 2019 were dramatically increased in 3.9, 2.7, and 2.8 times, respectively. Authors heavily imply that resistance surveillance on *M. genitalium* resistance mutations is of high importance and has to be included in clinical guidelines of the Russian Federation.

Code availability Not applicable.

Author contribution Study concept and design—Shedko E.D., Khayrullina G.A., Goloveshkina E.N.

Collection and processing of material—Shedko E.D., Khayrullina G.A.

Statistical analysis—Shedko E. D.

Writing—Shedko E.D.

Editing—Goloveshkina E.N., Akimkin V.G.

Approval of article final version—all co-authors.

Data availability All used reagent kits are commercially available.

Declarations

Ethics approval and consent to participate Retrospective analysis of previously obtained samples and cross-sectional examination does not require ethical review.

Consent for publication Not applicable.

Conflict of interest The authors declare that they have no conflict of interest.

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