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Research Article

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Evaluation of TIB Molbiol LightMix® assays for detection of *Mycoplasma genitalium* and key resistance mutations for macrolides and fluoroquinolones

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Abstract

The LightMix® Modular *Mycoplasma* Macrolide and LightMix® Modular *parC* Fluoroquinolone Resistance assays (TIB Molbiol) were evaluated using sequential *Mycoplasma genitalium* positive ($n = 125$) and negative ($n = 93$) clinical samples. Results were compared to the results of an established commercial assay (ResistancePlus MG assay, SpeeDx Pty Ltd) or Sanger sequencing (for *parC*). Detection of *M. genitalium* by the TIB Molbiol assay had a high agreement with the reference assay, with a positive percent agreement (PPA) of 97.6 [95% confidence interval (CI): 93.1–99.5] and negative percent agreement (NPA) of 95.7 (95% CI: 89.5–98.8). From 105 positive samples, macrolide resistance detection had a PPA of 100% (95% CI: 93.7–100) and NPA of 81.3% (95% CI: 67.4–91.1). For the detection of fluroquinolone resistance mutation G248T/S83I or "other mutation" in the quinolone resistance determinant region, from 95 samples there was 100% (95% CI: 86.3–100) sensitivity and 100% (95% CI: 94.5–100) specificity. The understanding of the basis for fluoroquinolone treatment failure is still developing; it is therefore important to use the output of *parC*-based resistance assays with caution to avoid the inappropriate use of antibiotic therapies, especially considering the limited number of alternative treatments.

Impact Statement

Assays detecting *Mycoplasma genitalium* antibiotic resistance can guide the choice of the most appropriate antibiotic for patient treatment. This study found that the LightMix® Modular *Mycoplasma* Macrolide and LightMix® Modular *parC* Fluoroquinolone Resistance assays (TIB Molbiol), performed well for *M. genitalium* detection and the detection of mutations associated with macrolide and fluoroquinolone failure. Due consideration should be applied to the results of such assays given the incomplete understanding of mechanisms of fluoroquinolone resistance.

Keywords: test method, microbial detection methods, PCR (polymerase chain reaction), bacterial infection, clinical microbiology

Introduction

Mycoplasma genitalium is a common bacterial sexually transmitted infection that causes nongonococcal urethritis in men and has been associated with adverse reproductive outcomes in women (Lis et al. 2015, Horner and Martin 2017, Noda-Nicolau et al. 2022). Given its small genome and the absence of a peptidoglycan-containing cell wall, the use of β-lactam and other glycopeptide antibiotics are ineffective in treating *M. genitalium* infection. With limited antimicrobial options, most international and national guidelines recommend the macrolide azithromycin for first-line treatment (Soni et al. 2019, CDC 2021, ASHM 2022, Jensen et al. 2022). However, recent analyses have highlighted significant increases in antibiotic resistance to macrolides, with point mutations in the 23S rRNA gene, conferring macrolide resistance and subsequent treatment failure with azithromycin (Machalek et al. 2020).

The fluoroquinolone moxifloxacin is often recommended for second-line treatment (Soni et al. 2019, CDC 2021, ASHM 2022, Jensen et al. 2022). Notably, difficulty in growing *M. genitalium* means that we still have an incomplete understanding of mechanisms of resistance, with a reliance on the analysis of clinical samples with known therapeutic outcomes. Failure with moxifloxacin is often associated with point mutations in a region of the *parC* gene referred to as the quinolone resistance-determining region (amino acids ~90–100) (Murray et al. 2022); specifically, these impact amino acid positions S83 and D87. In particular, the *parC* change G248T conferring amino acid change of S83I appears most relevant (Murray et al. 2020, 2023), with around 60% of cases with this mutation failing treatment (Murray et al. 2022).

Resistance-guided therapy has improved treatment outcomes for azithromycin treatment through the detection of mutations in the 23S rRNA gene (Read et al. 2019a). A similar approach to moxifloxacin treatment could theoretically lead to a >96% cure by diverting patients carrying G248T/S83I-positive bacteria to alternative therapies (Sweeney et al. 2022). Only a small number of noncommercial and commercial assays detecting *parC* variants have been published (Bodiyabadu et al. 2021, Shedko et al. 2021, Gardette et al. 2022, Li et

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al. 2022, Tickner et al. 2022, Chen et al. 2023). The aim of this study was to evaluate the TIB Molbiol modular assays LightMix® Modular *Mycoplasma* Macrolide and LightMix® Modular *parC* Fluoroquinolone Resistance.

Materials and methods

Sample collection

Sequential de-identified samples ($n = 218$, 93 diagnostically negative, 125 positive) that had been tested for *M. genitalium* (SpeeDx ResistancePlus® MG assay) were collected from a diagnostic molecular microbiology laboratory from July 2019 to October 2019. Samples were from a hospital clinic for women (providing contraceptive services and termination of pregnancy) and their male partners, plus external laboratories referrals for diagnostic testing (male and female). Sample types included 92 urine, 24 swabs (rectal, vaginal, and cervical), and 102 samples without a recorded specimen type. Patient demographic and clinical details were unavailable. This study used residual specimens and was approved as an audit by the Royal Women's Hospital Human Research Ethics Committee (AQA19/37); informed consent was not required.

Sample processing

Samples were extracted using the MagNA Pure 96 system utilizing the Viral NA Small Volume Kit and Pathogen Universal Protocol (eluted in 100 μl) as described previously (Murray et al. 2019). The TIB Molbiol extraction control PhHV was added to samples prior to extraction, as per manufacturer instructions.

Analysis by TIB MOLBIOL assays

DNA extracts were analysed using the STI panel kit [comprising TIB Molbiol Modular LightMix® assays: *Mycoplasma* Macrolide 53–0665-96 (LightCycler® 480 channel 510 nm), *Mycoplasma hominis* 61–0666-96 (610 nm), *Ureaplasma urealyticum/parvum* 64–0667-96 (640 nm), PhHV exogenous control 66–0901-96 (660 nm)] for the detection of *M. genitalium* and 23S rRNA gene mutation associated with macrolide resistance. Notably, the focus of this study is the detection of *M. genitalium* and associated markers of antibiotic resistance; performing the assay in an STI panel illustrates performance in the context of other detections. LightMix® Modular *parC* 53–0765-96 Fluoroquinolone Resistance was used for the detection of *parC* mutations associated with resistance to fluoroquinolones. Analyses were performed on the LightCycler® 480 II according to the manufacturer's instructions. Mutations associated with resistance were detected through melt curve analysis, with parameters described in Table 1.

Analysis by reference assays

Samples were tested on an established commercial assay (the reference assay) for the detection of *M. genitalium* and macrolide resistance mutations (ResistancePlus MG, SpeeDx Pty Ltd) according to the manufacturer's instructions. Results were analysed using UgenTec FastFinder version 3.5. Samples with discrepant results for macrolide resistance were further assessed via Sanger sequencing as described previously (Plummer et al. 2020). To evaluate the performance of LightMix® Modular *parC* Fluoroquinolone Resistance, results were compared directly to Sanger sequencing of the *parC* gene, performed as described previously (Plummer et al. 2020).

Table 1. Summary of melt analysis for LightMix® Modular kits.

Kit	Tm (°C)	Target/amino acid change	Clinical relevance
Mycoplasma Macrolide	66–68	Wild type 23S rRNA	
	<64	23S rRNA change	Macrolide resistant
parC	62.8	C234T/Wild type	Not relevant
	61.8	G248T/S83I	Fluoroquinolone resistant
	58.8	Wild type	Fluoroquinolone sensitive
	<58.0	Other QRDR mutations*	Variable

*These other quinolone resistance-determining region (QRDR) mutations include *parC* mutation/amino acid change G241T/G81S, G244A/D82N, A247C/S83R, A247T/S83C, G259A/D87N, G259T/D87Y, G259C/D87H, and A260G/D87G.

Table 2. Detection of *Mycoplasma genitalium* agreement for LightMix® STI Panel compared with the ResistancePlus® MG.

		LightMix® STI Panel 2		
		Pos	Neg	Total
ResistancePlus MG	Pos	121**	3*	124
	Neg	4*	90	94
	Total	125	93	218

*Samples with late Cp values.
**One sample previously identified as negative was positive with late Cp on both assays.

Table 3. Detection of *Mycoplasma genitalium* macrolide resistance mutations for LightMix® STI Panel compared with the SpeeDx ResistancePlus® MG.

		LightMix® STI Panel 2		
		MRM +	MRM −	Total
ResistancePlus MG	MRM+	57	0	57
	MRM−	9*	39	48
	Total	66	39	105

*8/9 samples were confirmed to contain a mutation that confers macrolide resistance by Sanger sequencing.

Data analysis

Positive percent agreement (PPA), negative percent agreement (NPA), and Cohen's kappa statistic were calculated for assay comparisons. Sensitivity and specificity were calculated for comparisons against *parC* sequence (gold standard).

Results and discussion

Agreement for the detection of *Mycoplasma genitalium*

From 218 samples (93 that were diagnostically negative, 125 positive), the LightMix® assay had a high level of agreement with an established diagnostic assay for the detection of *M. genitalium*, with PPA of 97.6% [95% confidence interval (CI): 93.1–99.5], NPA of 95.7% (95% CI: 89.5–98.8), and kappa of 0.934 (95% CI: 88.7–98.2) (Table 2). One sample was originally identified as negative by the diagnostic assay utilized for

Table 4. Detection of clinically relevant *parC* variants in *Mycoplasma genitalium* using LightMix® *parC* fluoroquinolone resistance compared to sequencing.

		LightMix® *parC*			
		G248T	Other mutation affecting ParC S83, D87	Not detected/irrelevant mutation detected^	Total
Sequencing	G259C (D87H)		1		1
	G259A (D87N)		2		2
	G248A (S83N)		2		2
	G248T (S83I)	19			19
	C234T/G248T* (S83I)		1		1
	WT^			70	70
	Total	19	6	70	95

*This mixed combination containing G248T/S83I was determined to be an "other mutant" by the LightMix® assay.
^This includes all mutations that do not confer an amino acid changes at S83 or D87, and are therefore associated with clinical failure of fluoroquinolones.

Table 5. Performance of alternative assays for the detection of *parC* mutations that may be associated with fluroquinolone resistance.

Assay	Company	Sensitivity (95% CI)	Specificity (95% CI)	Reference
MG *parC*	SpeeDx	98.2 (90.3–100)	99.3 (96.3–100)	Bodiyabadu et al. 2021
AmpliSens® *M. genitalium*-ML/FQ-Resist-FL	InterLabService Ltd	100 (90.8–100)	100 (98.4–100)	Shedko et al. 2021
Allplex MG & MoxiR Assay	Seegene	91.8 (83.2–96.2)	100 (97.7–100)	Gardette et al. 2022
MGMO qPCR	Nytor	94.4 (86.6–97.8)	100 (98.7–100)	Gardette et al. 2022

M. genitalium; however, both the reference assay (Resistance-Plus MG) and LightMix® STI panel 2 identified this sample as positive with late crossing point (Cp) values; this sample was not included in the macrolide resistance data analysis.

Detection of *M. genitalium* macrolide resistance mutations

For the analysis of macrolide resistance markers, there were 125 diagnostically positive samples; 5/125 (4%) samples were excluded from the analysis as the results were discordant for the presence of *Mycoplasma genitalium*, most likely due to low bacterial load, and a further 15/120 (12.5%) of samples had Cp values greater than the cut off for the LightMix® assay (i.e. the macrolide typing assay did not generate melt curves for resistance profiling). From the remaining 105 samples, there was complete PPA of 100% (95% CI: 93.7–100), NPA of 81.3% (95% CI: 67.4–91.1), and kappa of 0.825 (95% CI: 0.717–0.933) (Table 3). Analysis of discrepant samples found that the majority (8/9) deemed negative by the reference assay and positive by the LightMix® assay were indeed positive for macrolide mutation. Based on a composite result combining the diagnostic assays and sequencing, the prevalence of macrolide resistance mutations in the population was 65/105 (61.9%); this prevalence is similar to contemporary levels reported for women with *M. genitalium* in Melbourne, Australia (48%, for samples collected from 2017 to 2019) (Latimer et al. 2022) and for male samples (urine/urethral; 64%) and female samples (56%) in Queensland, Australia (Sweeney et al. 2019), but lower than levels found in men-who-have-sex-with-men (84%) (Read et al. 2019b).

Detection of *M. genitalium parC* variants

A total of 95 samples were analysed for the presence of mutation conferring fluoroquinolone resistance utilizing the LightMix® Modular *parC* Fluoroquinlone Resistance assay, and compared to Sanger sequencing of *parC* amplicons (Table 4). Overall, the sequencing determined prevalence of mutations affecting S83 was 22/95 (23.2%) and D87 was 3/95 (3.2%). The most clinically relevant variant G248T/S83I was present in 20/95 (21.1%) of samples. These proportions are similar to those previously reported for 2019–2020 in Melbourne, Australia, where 24.2% of samples from a sexual health clinic (including both male and female) had changes at *parC* S83 (including 23% conferring S83I) and 4.8% at *parC* D87 (Murray et al. 2022).

For the detection of mutations affecting key amino acids of ParC (S83 or D87), the LightMix® sensitivity was 25/25 (100%; 95% CI: 86.3–100), while specificity was 70/70 (100%; 95% CI: 94.5–100) and the kappa value was 1.00. For detection of the most clinically relevant mutation G248T/S83I, sensitivity was 95% (95% CI: 75.1–99.9), specificity 100% (95% CI: 95.2–100), and kappa of 0.968 (95% CI: 0.905–1), with one mutation missed in the presence of a second genetic variation.

Few commercial assays for the detection of *parC* mutations relating to fluoroquinolone resistance have been published, and each has received only limited clinical evaluation (Table 5) (Bodiyabadu et al. 2021, Shedko et al. 2021, Gardette et al. 2022). All have a high sensitivity/specificity. A previous evaluation of the TIB Molbiol assay found a similarly high level of performance with a sensitivity of 98.6% (95% CI: 92.4–99.8) and specificity of 95.4% (95% CI: 92.3–97.3) (Gardette et al. 2022).

Assay limitations

The load of *M. genitalium* in a sample can be very low, with as few as 10 bacteria per millilitre of urine (Murray et al. 2019). This can be problematic for polymerase chain reaction (PCR)-based antimicrobial resistance marker detection. Both assays used in this study were challenged by mutation discrimination in a minority of samples with low load (i.e. high Cp values), and it is beneficial to know where these limitations lie. One sample that was identified as having a change affecting ParC S83 or D87 by the LightMix® Modular *parC* Flu-

oroquinolone Resistance assay could not be fully genotyped due to the presence of two mutations, which confounded the melt curve analysis. The LightMix® *parC* assay has the benefit of discriminating the G248T/S83I mutation of *parC*, which is most clinically relevant and has the best diagnostic utility (Sweeney et al. 2022), and of being available on open PCR platforms. Of note, it has recently become evident that secondary mutations affecting *gyrA* may also contribute to fluoroquinolone resistance, although diagnostic assays targeting these mutations have not been developed (Murray et al. 2020, 2023).

Study limitations

The sample collection type was not recorded for all samples. Demographic information was unavailable, which may have led to the inclusion of a limited number of repeat tests and test of cure samples, impacting population estimates of mutations.

Conclusions

The TIB Molbiol LightMix® assays performed well for detection of *M. genitalium*, 23S rRNA gene macrolide resistance mutations, and *parC* mutations that contribute to fluoroquinolone resistance. While the utility of resistance-guided therapy for macrolide resistance is well established, an incomplete understanding of resistance mechanisms means that this approach should be used cautiously with fluoroquinolones to avoid the inappropriate use of fluoroquinolones, or alternative therapies. Current data suggest that *parC* G248T/S83I is the most important mutation, for both risk of failure and prevalence, while some of the other mutations affecting S83 and D87 do not appear to have a clear role in resistance.

Conflict of interest: This work was co-funded by TIB Molbiol. G.L.M. reports previously receiving funding support and diagnostic kits from Speedx Pty Ltd for research on *Mycoplasma genitalium* unrelated to this study.

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Author contributions

Prisha Balgovind (Formal analysis [equal], Investigation [equal], Methodology [equal], Project administration [equal], Writing – original draft [equal], Writing – review & editing [equal]), Steph Atchison (Formal analysis [equal], Investigation [equal], Methodology [equal], Writing – review & editing [equal]), Jennifer Danielewski (Methodology [equal], Supervision [equal], Writing – review & editing [equal]), Suzanne M. Garland (Resources [equal], Supervision [equal], Writing – review & editing [equal]), Anna-Maria Costa (Resources [equal], Writing – review & editing [equal]), Kaveesha Bodiyabadu (Investigation [equal], Methodology [equal], Writing – review & editing [equal]), and Gerald L. Murray (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Project administration [equal], Supervision [equal], Writing – original draft [equal], Writing – review & editing [equal])

Data availability

All data are presented in the document.

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