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Prevalence of *Mycoplasma genitalium* fluoroquinolone-resistance markers, and dual-class-resistance markers, in asymptomatic men who have sex with men

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Abstract

Introduction. Failure of fluoroquinolones, the principal treatment option for macrolide-resistant *Mycoplasma genitalium* infections, has recently emerged. This is of particular concern for men who have sex with men (MSM), who have high proportions of macrolide-resistant *M. genitalium* infections. Treatment failure with moxifloxacin is likely the result of single nucleotide polymorphisms (SNPs) in *parC*, whilst concurrent *gyrA* mutations may play a role.

Gap Statement. The levels of fluoroquinolone resistance and dual-class (i.e. macrolide and fluoroquinolone) resistance in *M. genitalium* among asymptomatic MSM is unknown.

Aim. To (i) determine the proportion of fluoroquinolone resistance and dual-class resistance in *M. genitalium* infections among asymptomatic MSM, (ii) explore any clinical and behavioural associations with fluoroquinolone resistance, and (iii) determine the distribution of antibiotic resistance among *M. genitalium mgpB* sequence types (STs).

Methodology. *M. genitalium* positive samples (*N*=94) were obtained from 1001 asymptomatic MSM enrolled in a study at Melbourne Sexual Health Centre (Carlton, Australia) between August 2016 and September 2017. Sanger sequencing was performed to determine the proportion of *M. genitalium* infections with SNPs in *parC* that have previously been associated with failure of moxifloxacin (corresponding to amino changes S83I, D83R, D87Y and D87N) and in *gyrA* (corresponding to amino acid changes M95I, D99N, D99Y and D99G). Associations between clinical/behavioural factors and *parC* SNPs were examined. Strain typing was performed by sequencing a portion of the *mgpB* gene.

Results. The proportion of MSM with infections harbouring *parC* and *gyrA* SNPs was 13.0% [95% confidence interval (CI): 6.8–23.2%] and 4.7% (95% CI: 1.1–13.4%), respectively; dual-class resistance was 13.0%. No significant clinical/behavioural associations were found. Antibiotic resistance was not restricted to specific *mgpB* STs.

Conclusion. One in eight (13%) of asymptomatic MSM with *M. genitalium* had an infection with dual-class-resistance mutations. Typing by *mgpB* sequence suggested fluoroquinolone resistance is arising from independent mutation events. This study illustrates that asymptomatic MSM may act as a reservoir for antibiotic-resistant *M. genitalium*.

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Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; MSHC, Melbourne Sexual Health Centre; MSM, men who have sex with men; SNP, single nucleotide polymorphism; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the *mgpB*, *gyrA* and *parC* sequences of the *Mycoplasma genitalium* isolates are MZ361843–MZ361913, MZ391894–MZ391957 and MZ391958–MZ392027, respectively.

One supplementary table is available with the online version of this article.

INTRODUCTION

Mycoplasma genitalium is a sexually transmitted bacterium associated with non-gonococcal urethritis in men, and cervicitis and pelvic inflammatory disease in women [1–3]. *M. genitalium* infections are commonly asymptomatic, with one study reporting a prevalence of 3% in asymptomatic men and 6% in asymptomatic women attending a sexual health clinic in the UK [4]. The prevalence of *M. genitalium* in asymptomatic men who have sex with men (MSM) has been reported to be 9.4–10.5% [5–7]. In MSM, anorectal infections are common and are often asymptomatic [8–14], with studies also reporting high levels of macrolide resistance in anorectal infections, between 75.0 and 84.2% [7, 12, 15]. Despite the high levels of anorectal infection, the contribution of rectal *M. genitalium* to proctitis is unclear [7, 10, 16, 17]. Furthermore, the contribution of asymptomatic infections to longer-term sequelae, such as prostatitis, epididymitis and balanoposthitis, is not fully understood [2].

The macrolide azithromycin has been commonly administered for treatment of *M. genitalium* infections, but has resulted in increased levels of macrolide resistance globally (10.0% before 2010 and 51.4% in 2016–2017) [18, 19]. Macrolide resistance is pronounced in MSM, with levels as high as 87.1% [20, 21]. Subsequently, the reliance on the use of fluoroquinolones for macrolide-resistant infections has increased, which may further increase the risk of fluoroquinolone resistance. A meta-analysis observed a global increase in fluoroquinolone-resistant *M. genitalium* from 4.8% (before 2010) to 9.3% (2016–2017) [18]. This is consistent with the results of an earlier review, which found a decrease in moxifloxacin efficacy from 100% (before 2010) to 89% (2010–2016) [22]. Studies have analysed the relationship between fluoroquinolone treatment failure and single nucleotide polymorphisms (SNPs) in *parC* and *gyrA* [23–26]. In *parC*, SNPs corresponding to the amino acid changes S83I [24–26], S83R [25, 26], D87N [23, 25, 26] and D87Y [23, 26] have been associated with fluoroquinolone failure. The contribution of *gyrA* SNPs alone is unknown; however, the presence of a *parC* S83I and a concurrent SNP in *gyrA* (conferring either M95I or D99N) may increase the risk of treatment failure [26].

Current guidelines recommend testing of *M. genitalium* in symptomatic individuals and their sexual partners [27]. Screening of asymptomatic individuals, especially MSM, is not recommended, as the contribution of *M. genitalium* to sequelae is unclear, and treatment may further exacerbate the issue of antibiotic resistance, particularly as treatment options are running out [28, 29]. MSM are a population who already experience high levels of macrolide-resistant *M. genitalium,* and any increase in fluoroquinolone resistance may result in dual-class-resistance strains, for which very limited treatment options are available [30]. Limited data are available on the trends and factors associated with fluoroquinolone-resistant *M. genitalium* in MSM [18, 31], particularly asymptomatic MSM. This study analyses clinical samples obtained from the MnM study of *M. genitalium* in asymptomatic MSM [7], and aims to (i) determine the levels of fluoroquinolone and dual-class resistance in the cohort, (ii) explore any clinical and behavioural factor associations with fluoroquinolone resistance in *M. genitalium*, and (iii) determine the distribution of antibiotic resistance among *M. genitalium mgpB* sequence types (STs).

METHODS

Sample collection

Samples were collected at Melbourne Sexual Health Centre (MSHC; Carlton, Australia) between 23rd August 2016 and 27th September 2017 in the MnM study, a study of *M. genitalium* in asymptomatic MSM [7]. Briefly, MSM were eligible to participate if they were ≥18 years of age, asymptomatic at both triage and clinical consultation, and reported receptive anal sex in the preceding year [7]. Urine and rectal swabs (either self- or clinician-collected) were screened for *M. genitalium* and macrolide resistance using the ResistancePlus MG test (SpeeDx). There was a total of 1001 participants, of which 95 (9.5%; 95% confidence interval [CI] 7.7–11.5%) were positive for *M. genitalium* and were recalled for treatment. Those who tested positive for rectal *M. genitalium* were asked to provide a throat swab for screening. Overall, 27 urine samples and 70 rectal samples tested positive for *M. genitalium*, with two participants providing both a urine and rectal *M. genitalium* sample (i.e. multi-site infection). Of the 70 MSM with rectal *M. genitalium,* 54 provided a throat sample of which 1 (1.9%) tested positive for *M. genitalium* [7]. All participants provided written informed consent and ethical approval was granted by the Alfred Hospital Ethics Committee (278/16).

Sequence analysis of samples

DNA extraction was performed in the parent study [7]. PCR amplification of *parC*, *gyrA* and *mgpB* was performed as described previously [32], with primers described elsewhere [26, 33]. The cycling conditions for *parC* and *gyrA* amplification were as follows: 98 °C for 30 s; followed by 35 cycles of 98 °C for 10 s, 57 °C for 10 s and 72 °C for 15 s; and final extension at 72 °C for 5 min. The cycling conditions for *mgpB* have been previously described [33].

Amplicons were sequenced bidirectionally at Macrogen. Sequences were processed using CLC Main Workbench (CLC Bio) and uploaded to GenBank with accession numbers MZ361843–MZ361913 (*mgpB*), MZ391894–MZ391957 (*gyrA*) and MZ391958–MZ392027 (*parC*). Some samples only had one sequence available for analysis (19 for *mgpB*, 1 for both *parC* and *gyrA*). SNPs were identified by MUSCLE alignment to the *M. genitalium* reference strain G37 (GenBank accession number NC_000908.2) using MEGA version 10.1.7 [34] and trimmed to equal length.

SNPs in *parC* and *gyrA* were considered to be clinically relevant if they had previously been associated with fluoroquinolone treatment failure. These include A247C/S83R

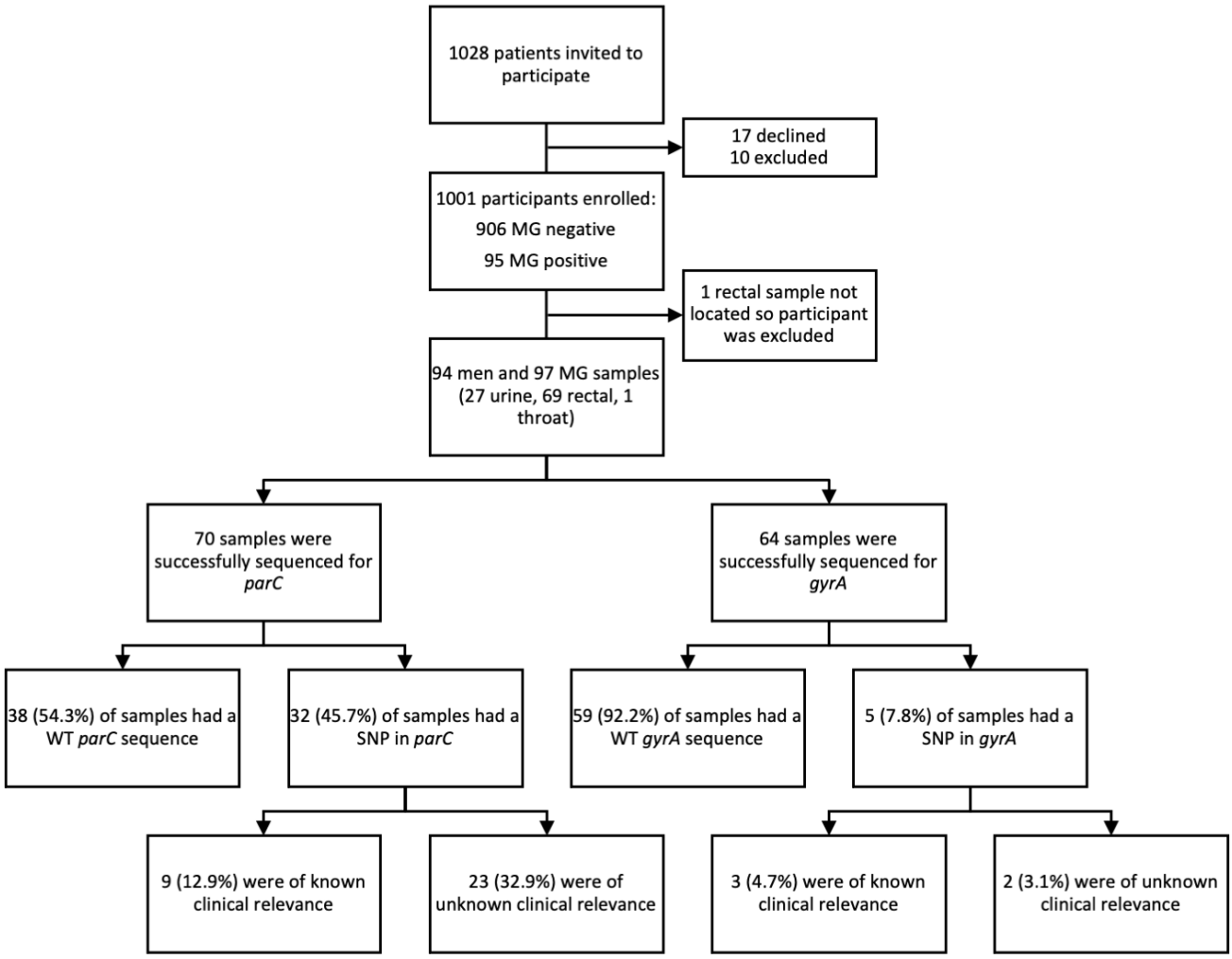

Fig. 1. Summary of the number of *M. genitalium* samples with wild-type (WT) or SNPs in *parC* and *gyrA*. Clinically relevant SNPs for *parC* were considered to be A247C/S83R, G248T/S83I, G259A/D87N and G259T/D87Y. Clinically relevant SNPs for *gyrA* were considered to be G285A/M95I, G295A/D99N, G295T/D99Y and A296G/D99G. MG, *M. genitalium*.

[25, 26], G248T/S83I [24–26], G259A/D87N [23, 25, 26] and G259T/D87Y [23, 26] for *parC*, and G285A/M95I [24–26], G295A/D99N [25, 26], G295T/D99Y [25, 26] and A296G/D99G [25] for *gyrA*. For *mgpB* typing, sequence data were uploaded to the Galaxy Australia web platform (https://usegalaxy.org.au) [35]. A maximum-likelihood phylogeny with 1000 bootstrap values was generated using IQ-TREE version 1.5.5.3 [36] and the best fit model was determined using Model Finder [37]. Phylogenetic trees were annotated using the online tool iTOL, version 5.6.1 [38].

Statistical analysis

Clinical and behavioural characteristics were obtained from questionnaires as part of the parent study [7]. A two-tail Fisher's exact test was performed to determine the association between characteristics and the detection of antibiotic-resistance-associated SNPs using Stata version 16.1 (StataCorp).

RESULTS

Study group

Of the 95 *M. genitalium*-positive men, one sample was not located; hence, 94 participants contributed a total of 97 *M. genitalium*-positive samples (27 urine, 69 rectal and 1 throat) (Fig. 1). The median age was 27 years (interquartile range: 23–32 years), most (94.7%) were human immunodeficiency virus (HIV) negative and were not on pre-exposure prophylaxis for HIV (PrEP) (80.9%). In the 3 months prior to enrolment, the majority of men reported inconsistent condom use (68.1%) and were not on antibiotics (70.2%). Nine men had prior exposure to azithromycin in the previous 3 months, while 18 men had exposure to other antibiotics not routinely used for *M. genitalium* treatment. Only one participant reported fluoroquinolone use (sitafloxacin) as part of resistance-guided therapy for a prior *M. genitalium* infection.

Table 1. Summary of *parC* and *gyrA* SNPs

SNP	Amino acid change	No. of samples (%)
parC*		
Clinically relevant		
G248T	S83I	7 (10.0)
G259A	D87N	1 (1.4)
G259T	D87Y	1 (1.4)
Not clinically relevant		
A147T	L49S	1 (1.4)
A154G	M52V	1 (1.4)
A173C	K58T	4 (5.7)
C179T	T60I	2 (2.9)
C184T	P62S	6 (8.6)
C234T	H78H	6 (8.6)
A282G	Q94Q	1 (1.4)
C324T	N108N	2 (2.9)
A346G	N116D	1 (1.4)
C356A	A119E	1 (1.4)
C356T	A119V	1 (1.4)
C366T	Y122Y	5 (7.1)
C375T	T125T	1 (1.4)
T424C	L142L	5 (7.1)
A436G	I146V	5 (7.1)
C438T	I146I	5 (7.1)
A454G	S152G	1 (1.4)
Total†		32/70 (45.7)
gyrA		
Clinically relevant		
G285A‡	M95I	3 (4.7)
Not clinically relevant		
T453C§	D151D	1 (1.6)
C468T‖	D156D	1 (1.6)
Total		5/64 (7.8)

*Some samples contained more than one *parC* SNP. The following combinations of *parC* SNPs were observed: G259T, C356T; C184T, A346G; C184T, C234T, T424C, A436G, C438T; A147T, A154G; C179T, G248T.
†One patient provided two *M. genitalium* samples, both contained *parC* C366T.
‡Detected with *parC* G248T (S83I).
§Detected with a group of *parC* SNPs (C184T, C234T, T424C, A436G, C438T).
‖Detected with *parC* C184T and A346G.

Proportion of samples with *parC* and *gyrA* mutations

Sequencing of *parC* was successful in 70 of 97 samples (72.2%). One participant had multiple samples sequenced (rectal and urine) and both samples had identical *parC* sequences containing a single SNP (C366T). Of the 69 individuals, 9 (13.0%, 95% CI 6.8–23.2%) had *M. genitalium* infections with *parC* SNPs/amino acid changes of clinical relevance (specifically G248T/S83I, G259A/D87N, G259T/D87Y), the most common being G248T/S83I (7/69, 10.1%) (Table 1).

For *gyrA*, 64 of the 97 samples (66.0%) were successfully sequenced and 3 of these (4.7%, 95% CI 1.1–13.4%) were *M. genitalium* infections with *gyrA* SNPs of known clinical relevance (G285A/M95I); all 3 also had a G248T/S83I *parC* change (Table 1).

Proportion of samples with dual-class-resistance-associated mutations

Of the 94 men with *M. genitalium* infections, 79 (84.0%, 95% CI 75.2–90.2%) had infections harbouring macrolide-resistance-associated mutations, as determined in the previous study [7]. All nine *M. genitalium* infections with clinically relevant *parC* SNPs had macrolide resistance detected; hence, dual-class-resistance infections were present in 13.0% (95% CI 6.5–22.3%) of the study group.

Associations between clinical/behavioural factors and fluoroquinolone resistance

Clinical and behavioural factors, including antibiotic usage in the previous 3 months, condom usage in the previous 3 months, clinical sample type, HIV status and the presence of co-infections, were analysed. No significant associations were observed in this study, likely due to the small sample size (Table S1, available with the online version of this article).

Sequence typing and distribution of antibiotic resistance among strains

Sequence typing of the *mgpB* gene was successful in 71 of 97 samples (73.2%). Overall, 24 *mgpB* STs were identified, of which 11 have previously been defined [39, 40] and 13 were undefined. The most common STs were ST-A/105 (21.1%) and ST-C/4 (21.1%). Macrolide resistance was present in the majority (63 out of 71, 88.7%) of samples and, therefore, was common to all STs identified in this study, while SNPs in *parC* and *gyrA* were not confined to specific STs (Fig. 2.).

DISCUSSION

This study analysed fluoroquinolone resistance in *M. genitalium* within an asymptomatic MSM population; 13.0% had a *M. genitalium* infection harbouring clinically relevant *parC* SNPs (specifically S83I, D87N, D87Y), and 4.7% with clinically relevant *gyrA* SNPs (M95I in conjunction with *parC* S83I). All infections with these *parC* SNPs were also macrolide resistant.

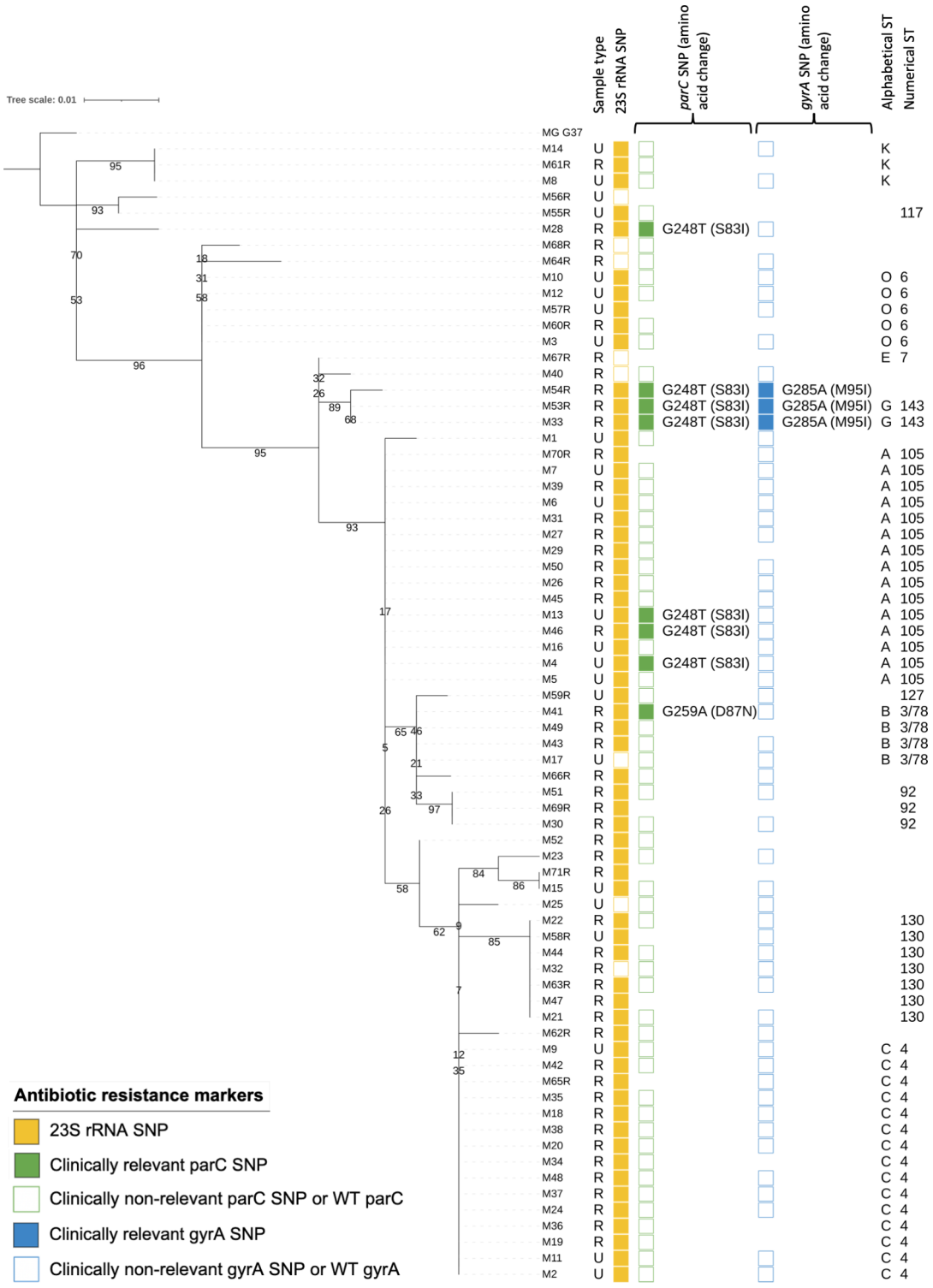

Fig. 2. Maximum-likelihood tree of 71 *mgpB* sequences generated using IQ-TREE version 1.5.5.3 (using Galaxy Australia) and reference sequence G37 (NC_000908.2) [35, 36]. The best-fit model was TPM2u+R3 and annotated using iTOL (version 5.6.1) [38]. The *mgpB* STs are labelled alphabetically [39] and numerically [40], and the absence of a ST label indicates an undefined ST. For 23S rRNA, *parC* and *gyrA* analysis, an open box indicates the sample did not have any key clinical SNPs, while a filled box indicates it contained a SNP of known clinical relevance. The absence of a box indicates no sequencing result was available for analysis. For sample type, U represents a urine sample, R represents a rectal sample. Sample names ending with 'R' indicate that only the reverse strand sequence was available for analysis. Bootstrap values are also displayed. The scale bar indicates the number of nucleotides substitutions per site.

As this study focused exclusively on asymptomatic MSM, there are limitations on the availability of similar studies for comparison. A study that examined the prevalence of *M. genitalium* in a group of predominantly asymptomatic MSM participants from an HIV study in France (from 2015 to 2016) reported a *parC* SNP prevalence of 9.1% (3/33), which is lower than the proportion observed in this study (13.0%) [6]. A study in Spain including asymptomatic MSM STI (sexually transmitted infection) clinic attendees (from 2017 to 2018) observed a similar prevalence of these *parC* SNPs, 13.2% (5/38) [41]. Dual-class resistance was not reported in either study. Collectively, these studies indicate that asymptomatic populations may act as an important reservoir for antibiotic-resistant *M. genitalium* infections, as these infections can be long duration and are unlikely to be identified and treated.

Another study from Belgium (from 2015 to 2018) examined levels of fluoroquinolone-resistance-associated mutations, but participants were not stratified by symptom status and either sex or sexual behaviour [31]. Both the levels of clinically relevant *parC* SNPs and dual-class resistance in asymptomatic male and female patients was 21.5% [31]. This is higher than the level observed in this study. The differences between levels of fluoroquinolone resistance may be due to the risk profile of the different study groups and their sexual behaviours, and differences in antibiotic prescribing between countries.

In a previous study of male and female attendees at MSHC from 2016 to 2018, testing of macrolide-resistant *M. genitalium* infections found *parC* SNPs in 21.2% (95% CI 17.1–26.0%) but similar levels of *gyrA* SNPs (5.6%, 95% CI 3.2–9.7%) [26]. Estimated levels of dual-class resistance were similar (16.4%) [26]. Notably, substantial fluctuation in the level of *parC* SNPs was observed over a 3 year period at MSHC, indicating that monitoring over longer trends is important to gain a clear picture [26].

Screening for *M. genitalium* in MSM is not recommended, as the natural history of infection and contribution to long-term sequelae are unclear [28]. Consequently, there is uncertainty around the need to treat asymptomatic MSM, particularly as extended-spectrum fluoroquinolones are costly, often hard to access in the community, and may cause severe side effects [7, 28]. However, asymptomatic carriage of *M. genitalium*, particularly at the rectum, and exposure to antibiotics for treatment of other sexually transmitted infections can contribute to the development and spread of resistant *M. genitalium* [7, 42]. The period of time for asymptomatic infections in men to resolve spontaneously is also unknown, but infections may persist for prolonged periods even after symptom resolution [2, 43]. Mathematical modelling indicates that screening of asymptomatic MSM may slightly reduce the prevalence and incidence of *M. genitalium*; however, treatment could potentially increase levels of macrolide resistance, particularly in settings where resistance-guided therapy is not available [44].

Diagnostic assays that report markers of fluoroquinolone resistance may assist in antibiotic prescribing and stewardship [29, 45]. The value of such assays in predicting treatment failure is still unclear, as not all patients with *M. genitalium* harbouring clinically relevant *parC* SNPs fail treatment [46]. This is largely because the molecular mechanism of fluoroquinolone resistance in *M. genitalium* is not fully understood, and other factors, such as organism load or pre-treatment with doxycycline, may influence treatment outcomes [25, 41]. More evidence is needed regarding the need to screen for *M. genitalium*, and the role of quinolone-resistance assays in clinical care.

Phylogenetic analysis revealed that antibiotic-resistance-associated SNPs varied within groups of *mgpB* STs. This suggests that transmission is not the sole cause of antibiotic-resistance spread in this study group; rather, *parC* SNPs have likely arisen independently during fluoroquinolone treatment. Similar findings were reported in studies by Fernandez-Huerta *et al.* (2020) and Sweeney *et al.* (2020) where they both observed *de novo* acquisition of *parC* mutations [47, 48]. Insufficient information was available to analyse the distribution of *gyrA* SNPs.

The most common STs observed in this study were ST-A/105 and ST-C/4. Of these, ST-C/4 has been frequently reported in *mgpB* typing studies from Spain, France and Germany [48–51]. In this study, only three samples belonging to ST-A/105 harboured a *parC* SNP (G248T/S83I) and no *gyrA* SNP, while samples from ST-C/4 had neither a *parC* nor *gyrA* SNP. Analysis of globally diverse samples should be performed to examine the full diversity of *M. genitalium* and to further examine the distribution of antibiotic resistance among *M. genitalium* strains.

This study has a number of limitations, including a small sample size that precluded some statistical analyses, and sequencing was unsuccessful in a proportion of samples (likely due to low organism load). Clinically relevant SNPs were reported based on previous studies [23–26], but whether these mutations confer fluoroquinolone resistance is still unknown, due to the difficulty of performing antimicrobial-susceptibility testing. Analysis of additional loci could be performed to increase the discriminatory power of the strain typing; however, with no association found between ST and antibiotic-resistance mutations, this would not have changed the outcome of the study. Moreover, the results in this study may not be reflected in other asymptomatic MSM populations, especially in settings where antibiotic consumption differs. Examination of transmission between participants and their sexual partners could not be performed; hence, whether asymptomatic MSM are truly a reservoir for antibiotic-resistant *M. genitalium* could not be determined in this study. Future studies should be undertaken to examine this.

In conclusion, this study describes a concerning level of *M. genitalium* fluoroquinolone and dual-class-antibiotic resistance in asymptomatic MSM. This may form a hidden reservoir of antibiotic resistance that may contribute to community spread of antimicrobial resistance in *M. genitalium*.

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Conflicts of interest
G.L.M., C.S.B. and S.M.G. have jointly received grant funding with SpeeDx Pty Ltd.

Ethical statement

All participants provided written informed consent and ethics was approved by the Alfred Hospital Ethics Committee (278/16).

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