Antimicrobial resistance of *Neisseria gonorrhoeae* isolates from high risk men in Johannesburg, South Africa

Authors

Liteboho D. Maduna[a], Marleen M. Kock[a,b], Brian M. J. W. van der Veer [c], Oscar Radebe[d,e], James McIntyre[d], Lieke B. van Alphen[c], Remco P.H. Peters[a,c,d,f]#

Affiliations

a. Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa
b. Tshwane Academic Division, National Health Laboratory Service, Pretoria, South Africa
c. Department of Medical Microbiology, School of Public Health & Primary Care (CAPHRI), Maastricht University Medical Centre, Maastricht, The Netherlands
d. Anova Health Institute, Johannesburg, South Africa
e. EpiC Family Health international (FHI360), Pretoria, South Africa
f. Foundation for Professional Development, Research Unit, East London, South Africa

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#Address for correspondence

Prof R.P.H. Peters: University of Pretoria, Faculty of Health Sciences, Department of Medical Microbiology, Pathology building, Room 3-11, Pretoria 0001, South Africa. Email: rph.peters@gmail.com.

ABSTRACT

Objectives. *Neisseria gonorrhoeae* antimicrobial drug resistance has emerged worldwide, however, the situation in Sub-Saharan Africa is not well-documented. We investigated the molecular epidemiology and occurrence of antimicrobial resistance in *Neisseria gonorrhoeae* infections in two core transmission groups of men in Johannesburg, South Africa.

Methods. We recruited men who have sex with men (MSM) presenting with urethral discharge and men with a recurrent episode of urethral discharge. Molecular testing and culture for *N. gonorrhoeae* followed by antimicrobial susceptibility testing was performed. Whole genome sequencing (WGS) was used to identify resistance conferring mutations and to determine genetic relatedness of the isolates.

Results. Fifty-one men were recruited; 42 (82%) had *N. gonorrhoeae* infection. Most gonococcal isolates were resistant to ciprofloxacin (78%) and tetracycline (74%); 33% were penicillin resistant. All gonococcal isolates were susceptible to cephalosporines and spectinomycin. Azithromycin resistance was observed in four (15%) isolates (epidemiological cut-off); all with mutations in the *mtrR* promoter region. Most of the isolates (19/27) harboured the gonococcal genetic island; associated with antimicrobial resistance. WGS revealed a diverse epidemic with mostly novel NG-STAR (70%) and NG-MAST (70%) sequence types.

Conclusions. We demonstrate high prevalence of antimicrobial-resistance in *Neisseria gonorrhoeae* strains obtained from high-risk men in South Africa. Introduction of diagnostics and scale-up of surveillance are warranted to prevent emergence of multidrug-resistant infections.

Keywords. *Neisseria gonorrhoeae*, core transmission groups, whole genome sequencing, antimicrobial resistance, azithromycin, ceftriaxone, ciprofloxacin

INTRODUCTION

Gonorrhea is a major public health concern globally; the World Health Organization (WHO) estimated that 87 million new infections occurred among 15-49-year olds in 2016, with the highest incidence found in Sub-Saharan Africa (1, 2). *Neisseria gonorrhoeae* is included in the WHO global priority list of antibiotic-resistant bacteria as it has developed resistance to every antimicrobial drug recommended for treatment since the introduction of the sulphonamides in the 1930s (3, 4).

The recent emergence of gonococcal strains in Australia and United Kingdom resistant to ceftriaxone and azithromycin, has raised major concerns of untreatable of gonorrhea (5). However, there are limited data available on resistance of *N. gonorrhoeae* strains circulating in Sub-Saharan Africa, the region with the highest burden of infection, and their mechanisms of antimicrobial resistance (2-4, 6). This paucity of data is due to the lack of access to laboratory diagnostic services and the use of syndromic management for treatment of sexually transmitted infections (STIs) in this region (7). Syndromic management has many limitations including lack of susceptibility testing, inability to identify asymptomatic infections and limited opportunity for widespread surveillance, and data on individuals presenting with treatment failure are not recorded (8, 9). Current syndromic management in South Africa consists of 1 gram azithromycin with 250mg ceftriaxone (10).

Core transmission groups such as men who have sex with men (MSM) and those with recurrent episodes of gonorrhea have played an important role in the emergence of drug resistance (11). In South Africa and elsewhere in Africa (12, 13), there a is a high burden of *N. gonorrhoeae* infections among MSM (14, 15); the first two cases of cefixime-resistant *N. gonorrhoeae* infections in Africa were reported in this population (16). It is therefore imperative to understand the drug resistance profile of gonococcal populations in core

transmission groups, and to include these populations in sentinel surveillance to inform clinical management guidelines and policy design (17).

Whole genome sequencing (WGS) provides a high resolution molecular epidemiological tool for describing gonococcal populations and their mechanisms of antimicrobial resistance (18,19). However, conducting quality-assured WGS studies of *N. gonorrhoeae* in low-resource settings may be challenging and is rarely conducted due to poor laboratory infrastructure and the prolonged time required for specimens to reach the laboratory for culture (7, 20). In addition, sentinel surveillance is limited due to a lack of resources and generally only includes the heterosexual population.(7, 20). Nevertheless, molecular epidemiological studies are essential to map the status of antimicrobial resistance in *N. gonorrhoeae* infections in sub-Saharan Africa and to monitor for emergence of resistance.

We conducted a cross-sectional study to assess the antimicrobial resistance profiles and determine the molecular epidemiology of *N. gonorrhoeae* in two core groups of high-risk men visiting sexual health services in Johannesburg, South Africa.

MATERIALS & METHODS

Study Design and Setting

This cross-sectional study was conducted at three primary healthcare facilities (PHC) offering health services to men in Johannesburg, South Africa, between March 2018 and April 2019. One facility is in the city centre and the other two are in Soweto, Johannesburg's largest township. Most of the clients visiting these facilities are of low socio-economic status (21). The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, South Africa (Ref 253/2017) and the Johannesburg District Research Committee.

Study population

We recruited two groups of men (>18 years old) at high risk of *N. gonorrhoeae* infection: a) men with recurrent or persistent discharge within one month of initial treatment as provided by a healthcare facility in the public or private sector in South Africa, and b) MSM (born male with any type of sexual contact with other men in the preceding six months) presenting with urethral discharge. Written informed consent was obtained from all participants.

Study Procedures

Physical examination was conducted, and questionnaires were administered in a face-to-face interview to collect data on demographics and recent sexual behaviour. Study nurses collected urethral swabs (Copan Diagnostics, Italy), which were immediately inoculated on New York City (NYC) agar medium at the study site followed by transport in an AnaeroPack^{TM} (Thermo Scientific, Lenexa, USA) to the Department of Medical Microbiology at the University of Pretoria for further processing. First-void urine was collected for molecular detection of *N. gonorrhoeae, Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium* infection. Participants were provided with standard syndromic management upon recruitment; targeted follow-up treatment was provided if indicated based on laboratory result (10).

Microscopy, culture and antimicrobial susceptibility testing

The inoculated NYC agar plates were incubated at 37 °C with 5% CO_2 at the Department of Medical Microbiology, University of Pretoria, and were assessed for microbial growth daily. Presumptive *N. gonorrhoeae* colonies on NYC agar plates were positively identified using, Gram stain, rapid oxidase test and API NH (BioMerieux, France). This was followed by antimicrobial susceptibility testing to ciprofloxacin, azithromycin, ceftriaxone, cefixime, penicillin G, tetracycline and spectinomycin using E-tests (bioMérieux, France) following

manufacturer's instructions. Minimum inhibitory concentrations (MICs) were interpreted according to European Committee of Antimicrobial Susceptibility Testing breakpoints (EUCAST) except for azithromycin where the epidemiological cut-off (ECOFF) value was used as no resistance breakpoint exists (www.eucast.org). The *N. gonorrhoeae* ATCC 49266 and 19424 strains were used as quality control.

Molecular detection of pathogens causing male urethritis syndrome

Urine specimens were tested for presence of *N. gonorrhoeae* and *C. trachomatis* DNA using Lightmix Kit 480 HT CT/NG assay (TIB MOLBIOL, Berlin, Germany). The presence of *M. genitalium* and *T. vaginalis* DNA were tested for with validated in-house real time polymerase chain reaction (PCR) as described elsewhere (22, 23). Additionally, *C. trachomatis* positive samples were tested for the lymphogranuloma venereum (LGV) biovar as previously described and *M. genitalium* positive specimens were assessed for presence of macrolide resistance (24, 25).

DNA preparation and whole genome sequencing

Genomic DNA was prepared from single colonies using High Pure PCR Template Preparation kit (Roche, Germany). *N. gonorrhoeae* DNA sequencing library was prepared using the NexteraXT library preparation kit (Illumina, Eindhoven, the Netherlands). Paired-end 250-bp indexed reads were generated on the Illumina MiSeq instrument following manufacturer's instructions (Illumina, Eindhoven, the Netherlands). In brief, raw reads were assessed for quality using FastQC (26), low-quality bases and adaptor sequences were trimmed with Trimmomatic (27). Contaminants were investigated using Kraken v2.0.7 and removed using DeconSeq v.4.3 (28, 29). Raw reads were de novo assembled using SPAdes v.3.9.0 and quality

was assessed using Quast v4.3 (30, 31). WGS read data was submitted to National Centre for Biotechnology Information under BioProject accession No PRJNA575338.

Molecular epidemiological analysis

Core genome single nucleotide polymorphisms (SNPs) were determined using ParSNP v1.2 using the NCCP 11945 reference strain (GenBank accession number CP001050.1) (32). Ten additional strains from diverse international locations, including strains WHO-F, WHO-G, WHO-L, WHO-M, WHO-N, WHO-O, WHO-P, WHO-U, 46146 (Kenya) and 46745 (Kenya) (6, 33) were included in this analysis to provide a variety *N. gonorrhoeae* multiantigen sequencing typing (NG-MAST) and multilocus sequence typing (MLST) sequence types (STs) and geographical spread of the isolates. Recombination blocks were removed using Gubbins v1.4.10 and maximum-likelihood phylogenetic tree was generated with RAxML v8.2.9 with GTRCAT substitution model (34, 35). Phylogenetic trees were visualized and annotated using Interactive Tree of Life (36).

For analysis of genes implicated in drug resistance, we used the *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) database which utilises DNA sequences of seven known antimicrobial resistance (AMR) determinants (*penA, mtrR, porB, ponA, gyrA, parC* and 23S rRNA) and the genome comparator tool at Bacterial Isolate Genome Sequence (BIGSdb) (https://pubmlst.org/neisseria); which defines chromosomal and plasmid genes implicated in gonococcal AMR (37, 38). New alleles and allele combinations were submitted to https://ngstar.canada.ca for assignment of new sequence types (STs). The presence of the gonococcal genetic island (GGI), a type 4 secretion system implicated in horizontal gene transfer (39) ; was investigated using the BIGSdb.

For the molecular epidemiology of the isolates, BIGSdb (http://pubmlst.org/neisseria/) was used to determine the MLST and NG-MAST STs. The *N. gonorrhoeae* MLST measures

DNA sequence variations in a set of seven housekeeping genes (*abcZ, adk, aroE, fumC, gdh, pdhC,* and *pgm*) and characterizes strains by their unique allelic profiles (38). The NG-MAST analyses two hypervariable loci of *N. gonorrhoeae*; *porB* (490 bp) and *tbpB* (390 bp) (38). All novel alleles were submitted to the curator for assignment of allelic numbers and STs. NG-MAST STs which were yet to be assigned STs were inferred manually using a unique number. In case of positive molecular results for *N. gonorrhoeae* but no growth in culture, culture-free NG-MAST was performed on non-culture viable PCR positive *N. gonorrhoeae* clinical samples as previously described (40).

Statistical analysis

Descriptive data are provided as number with proportion and median with range. Comparison of categorical variables between groups was done using the Chi-squared test with Fisher's Exact test when appropriate.

RESULTS

Characteristics of the study population

We recruited a total of 51 men in the study; 31 were from the two township clinics and 20 from the inner-city clinic. The median age was 27 years (range 22-38 years) and seven men (14%) were HIV-infected of whom six (86%) men were on antiretroviral therapy **(Table 1)**. More than half of the men (n=31; 61%) reported discharge that their discharge was recurrent or persistent following recent treatment. At enrolment, 21 (41%) men identified as homosexual, 11 (22%) bisexual and 19 (37%) heterosexual. Overall, most participants 44 (86%) reported multiple sex partners in the previous six months and three-quarters of them [38 (75%)] had been treated for an STI in the previous six months.

Table 1. Demographic characteristics of the study population (n=51)

Characteristics	MSM with urethral discharge (n= 20)	MSM repeat STI symptoms (n= 12)	Heterosexual men with repeat STI symptoms (n=19)
	n (%)	n (%)	n (%)
Employment status			
Self-employed	1 (5)	0 (0)	2 (11)
Formal employment	12 (60)	5 (42)	9 (47)
Unemployed	6 (30)	4 (33)	8 (42)
Student	1 (5)	3 (25)	0 (0)
Sexual orientation			
Heterosexual	0 (0)	0 (0)	19 (100)
Homosexual	15 (75)	6 (50)	0 (0)
Bisexual	5 (25)	6 (50)	0 (0)
Sexual practice in last 6 months			
Sex with men only	15 (75)	6 (50)	0 (0)
Sex with females only	0 (0)	0 (0)	19 (100)
Sex with both men and women	5 (25)	6 (50)	0 (0)
Condom use last sex act			
Yes	4 (20)	1 (8)	2 (11)
No	16 (80)	11 (92)	17 (89)
Multiple sex partners in last 6 months			
Yes	15 (75)	10 (83)	19 (100)
No	5 (25)	2 (17)	0 (0)
Treated for any STI-associated symptoms in the past 6 months			
Yes	7 (35)	12 (100)	19 (100)
No	13 (65)	0 (0)	0 (0)

HIV status at enrolment			
Positive	2 (10)	4 (33)	1 (5)
Negative	18 (90)	8 (67)	16 (84)
Unknown	0 (0)	0 (0)	2 (11)

Data are presented as numbers with proportion.

Abbreviations: MSM, men who have sex with men; STI, sexually transmitted infection; HIV, Human immunodeficiency virus.

Etiology of male urethritis syndrome at enrolment

An STI was detected in urine specimens of 90% (46/51) of men: 82% (42/51) participants had *N. gonorrhoeae* infection followed by *C. trachomatis* (11/51; 22%), *M. genitalium* (10/51; 20%) and *T. vaginalis* (4/51; 8%) **(Table S1)**. Twenty-one men (41%) had more than one STI detected. *N. gonorrhoeae* was more commonly detected among MSM (n=29/32; 91%) compared to heterosexual men (n=13/19; 68%) with recurrent or persistent discharge p=0.04. All 11 *C. trachomatis* positive specimens tested negative for the LGV biovar. *M. genitalium* macrolide resistance associated mutation in the 23S rRNA (A2071G) and a *parC* mutation (Pro-62→Ser) associated with quinolone resistance were detected in one case (41).

Antimicrobial susceptibility testing

Gonococcal cultures were positive for 27/42 men (64%) with *N. gonorrhoeae* detected molecularly. Eight (30%) of the cultured isolates demonstrated phenotypical resistance to three or more antimicrobial drugs and could be classified as multidrug-resistant (MDR) *N. gonorrhoeae* (42). Antimicrobial susceptibility data and molecular markers associated with resistance of *N. gonorrhoeae* are presented in **Tables 2 and 3**.

Table 2. Antimicrobial susceptibility profiles and minimum inhibitory concentrations of *Neisseria gonorrhoeae* isolates collected from men in South Africa (n=27)

Drug	Susceptible* n (%)	Resistant n (%)	Median MIC value	MIC range (mg/L)
Ciprofloxacin	6 (22)	21 (78)	0.75	0.002-4
Azithromycin*[a]	23 (65)	4 (15)	0.25	0.023-8
Penicillin	18 (67)	9 (33)	0.5	0.016-12
Tetracycline	7 (26)	20 (74)	8	0.125-96
Spectinomycin	27 (100)	0	6	4-48
Cefixime	27 (100)	0	0.016	0.016-0.023
Ceftriaxone	27 (100)	0	0.002	0.002-0.004

Abbreviation: MIC, minimum inhibitory concentration.

*EUCAST breakpoints were used to classify strains as susceptible or resistant.

[a]no EUCAST resistance breakpoint exists and instead the epidemiological cut-off (ECOFF) value of 1.0 mg/L was used for interpretation.

Most of the isolates were phenotypically resistant to ciprofloxacin (n=21; 78%). The following mutations in the GyrA were identified: 21 (100%) Ser-91→Phe, 13 (62%) Asp-95→Gly and 8 (38%) Asp-95→Ala. ParC mutations were observed in 16 isolates of which 15 were ciprofloxacin resistant based on phenotypic testing.

Azithromycin resistance interpreted using ECOFF, was identified in 4/27 (15%) gonococcal isolates (MIC range 1-8 mg/L); all had been obtained from MSM. The *mtr*R promoter adenine deletion (-35A del) was identified in two isolates (MICs 4 and 8 mg/L) and an Ala-39→Thr mutation in the MtrR repressor was identified in the other two (MIC of 1 mg/L). Mutations in the MtrR repressor which regulates MtrCDE efflux pump are associated with overproduction of the *mtrCDE* efflux operon known to export macrolides (43). An Ala-39→Thr mutation was identified in 6/27 azithromycin isolates classified susceptible as per ECOFF (MIC range 0.5-0.75 mg/L). No known mutations in the 23S rRNA and *macAB* promoter that confer resistance to macrolides were detected.

Table 3. Resistance-associated mutations identified in *Neisseria gonorrhoeae* isolates resistant to penicillin, ciprofloxacin, tetracycline, azithromycin and tetracycline

Drug	Resistant isolates (n)	Isolates with molecular resistance determinants (n)	Protein	Mutation	Isolates with this gene/ mutation n (%)
Ciprofloxacin	21	21	GyrA	Ser-91→Phe	21 (100)
				Asp-95→Gly	13 (62)
				Asp-95→Ala	8 (38)
			ParC	Ser-87→Ile	2 (10)
				Ser-87→Asn	6 (29)
				Glu-91→Gly	1(5)
				Asp-86→Asn	6 (29)
Azithromycin	4	4	*mtrR*-promoter	-35A del	2 (50)
			MtrR	Ala-39→Thr	2 (50)
Penicillin	9	9	PonA	Leu-421→Pro	9 (100)
			PorB1b	Gly-120→Lys & Ala-121→Asp	1(11)
				Gly-120→Asn & Ala-121→Asn	1 (11)
				Ala-121→Ser	1 (11)
			mtrR- promoter	-35A del	1 (11)
			MtrR	Ala-39→Thr	4 (44)
			$bla_{\text{TEM-1B}}$	-	8 (89)
			$bla_{\text{TEM-135}}$	-	1 (11)

Tetracycline	20	20	PorB	Gly-120→Lys & Ala-121→Asp	2 (10)
				Gly-120→Asn & Ala-121→Asn	1 (5)
				Ala-121→Ser	6 (30)
			mtrR promoter	-35A del	2 (10)
			MtrR	Ala-39→Thr	10 (50)
			tetM (Dutch)	-	5 (25)
			tetM (American)	-	15 (75)
			RpsJ	Val-57→Met	14 (70)

There was no resistance or decreased susceptibility to cefixime or ceftriaxone among our isolates, but one-third of isolates (9/27; 33%) demonstrated resistance to penicillin. All resistant isolates had the Leu-421→Pro mutation in the penicillin-binding protein (PBP1) associated with penicillin resistance (44). Also, the following PorB1b mutations were identified: Gly-120→Lys & Ala-121→Asp (1/9), Gly-120→Asn & Ala-121→Asn (1/9) and Ala-121→Ser (1/9). Furthermore, 4/9 isolates had the Ala-39→Thr mutation in the MtrR repressor associated with overexpression of MtrCDE efflux pump. An adenine deletion (-35A del) in the promoter region of *mtrR* was identified in one gonococcal isolate. Eight out of nine penicillin resistant isolates harboured the $bla_{\text{TEM-1}}$ plasmid; the ninth had $bla_{\text{TEM-135}}$ (MIC= 12mg/L) associated with resistance to β-lactams (45).

Most of the isolates, 20/27 (74%) were phenotypically resistant to tetracycline. All tetracycline-resistant isolates harboured a conjugative plasmid *tetM*: 15 (75%) were of American and 5 (25%) of the Dutch type. Five isolates demonstrated high level plasmid-mediated tetracycline resistance (MIC ≥ 16 mg/L); all harboured American-type *tetM* plasmid.

Two (10%) isolates had the -35A del in *mtrR* promoter, and 10 isolates (50%) had the Ala-39→Thr mutation, whereas a Val-57→Met mutation in the ribosomal protein s10 (RpsJ) was detected in 14 (70%) of the 20 tetracycline-resistant gonococcal isolates. Most of the isolates, 19/27 (70%) harboured the GGI, a type 4 system implicated with AMR to multiple antimicrobials (39). We did not observe any resistance to spectinomycin.

Molecular epidemiology and phylogenetic analysis

Phylogenetic analysis of the WGS data of the 27 gonococcus isolates revealed a large diversity among *N. gonorrhoeae* strains. The gonococcal strains were interspersed across the phylogenetic tree, they did not cluster based on the location where the isolates were collected, sexual orientation of the participants or antimicrobial resistance profiles (**Figure 1**). A phylogenetic comparison of the gonococci from South Africa with isolates from coastal Kenya and WHO reference strains revealed gonococci from South Africa were distinct as they did not cluster with gonococci from international locations (**Figure 1**).

Neisseria gonorrhoeae sequence typing for antimicrobial resistance (NG-STAR) analysis identified 24 different STs; 19 (70%) were novel (**Table S2**). Five strains with novel NG-STAR STs had novel *mtr*R alleles whereas NG-STAR ST1942, ST1935 and ST1930 possessed novel *parC* alleles.

Multilocus sequence typing (MLST) identified 18 different STs of which five were new to the PubMLST database. The most frequently identified sequences types were ST1588 (n=3), ST1893 (n=3), ST13942 (n=3) and ST1579 (n=2).

In silico analysis of WGS data classified 27 isolates into 24 different NG-MAST STs. Most of these STs (n=19; 70%) have not been previously described; we identified seven novel *porB* and nine novel *tbp*B alleles. We further employed culture-free NG-MAST on the 15 *N. gonorrhoeae* PCR positive specimens without growth in culture. Ten (67%) clinical samples

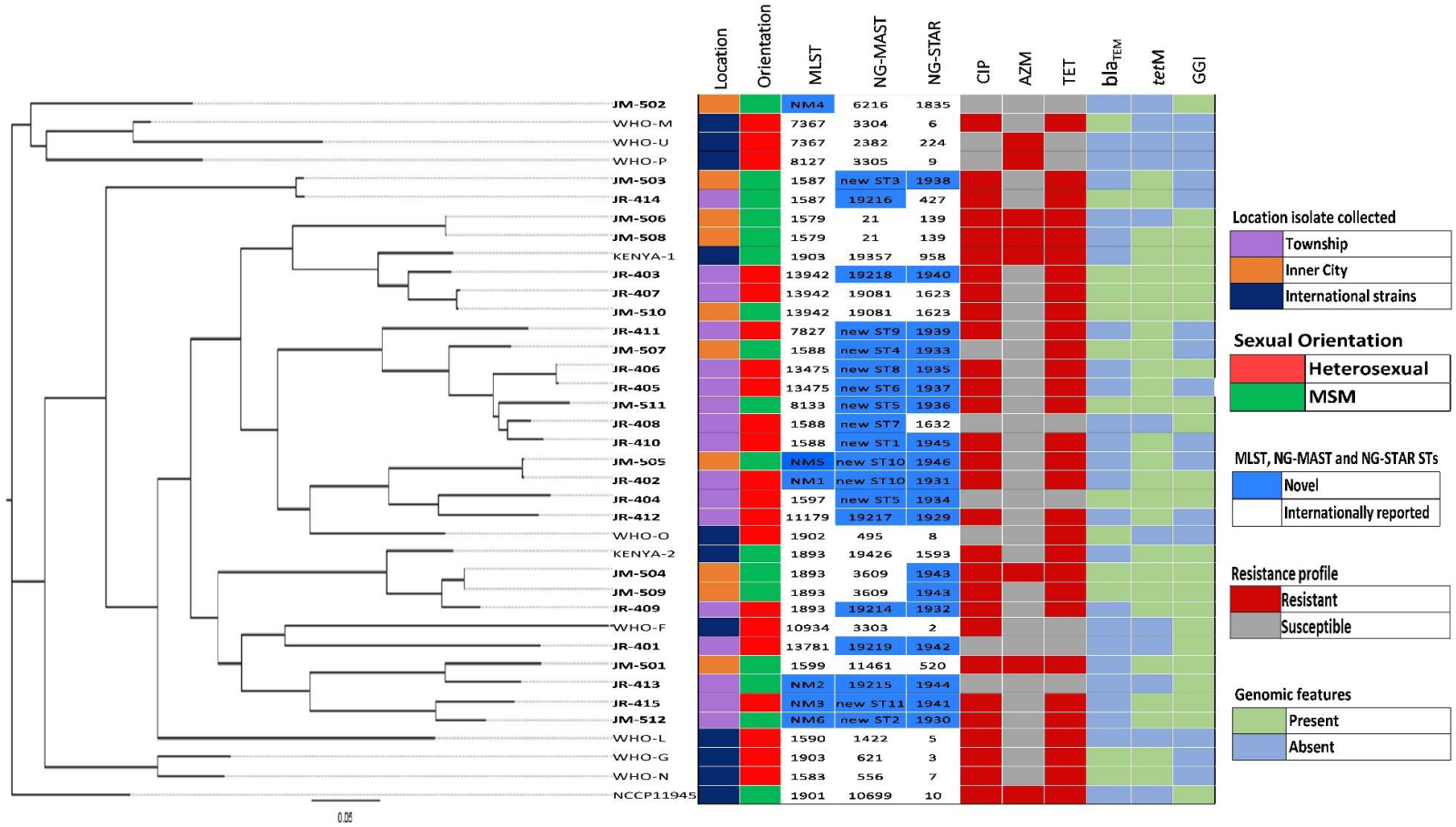

Figure 1. Whole-genome core single-nucleotide polymorphism maximum likelihood phylogenetic tree of 27 *N. gonorrhoeae* strains obtained in South Africa including 10 international strains and the NCCP11945 reference strain. The length scale bar represents the estimated evolutionary divergence between isolates. Location where isolates were obtained, sexual orientation, multilocus sequence type (MLST), multiantigen sequence type (NG-MAST) and *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) are indicated. Susceptibility to ciprofloxacin (CIP), azithromycin (AZI) and tetracycline (TET) resistance shown. The presence bla_{TEM} gene, *tet*M gene and the gonococcal genetic island is indicated. Novel sequence types are indicated.

were successfully genotyped resulting in both *porB* and *tbpB* sequences and these were allocated an NG-MAST ST; for 5 specimens we only managed to generate *porB* but not *tbpB* results. The NG-MAST dendrogram (**Figure S1**) confirms the genetic diversity observed in WGS; there is no clustering of NG-MAST STs of strains that were successfully cultured and those that were uncultured but typed successfully by culture-free NG-MAST.

DISCUSSION

This study is among the first in Africa to provide a comprehensive in-depth WGS analysis of *N. gonorrhoeae* infection among core transmission groups that are generally associated with emergence of antimicrobial resistance. We have demonstrated that urethral gonococcal infection and antimicrobial resistance are highly prevalent in this selected population of high-risk men in South Africa. We also demonstrate that, in this population, there is a distinct epidemic of *N. gonorrhoeae* strains circulating that is genetically unique based on the high number of novel STs identified.

As expected from other studies, *N. gonorrhoeae* infection was identified as the main aetiological cause of urethral discharge in men in our study (12, 13). Most participants reported unprotected sex and multiple sex partners; the coinfection rate with other STIs was high confirming that we recruited a core transmission group of high-risk men (13, 46). Although our study cohort may not be fully representative of the general male population, it constitutes an important sentinel population to study the emergence of *N. gonorrhoeae* resistance and the potential for epidemic spread (4). One-third of the isolated *N. gonorrhoeae* strains in our study was classified as MDR and high rates of ciprofloxacin and tetracycline resistance were observed; these drugs were both used in syndromic management of STIs for a prolonged period of time in South Africa; ciprofloxacin was only discontinued in 2008 and doxycycline in 2015 (47). These high rates of antimicrobial resistance for these two drugs are in line with reports

from national surveillance and another recent study conducted in the KwaZulu-Natal Province in South Africa (47, 48). The cumulative mutations in the quinolone resistance determining regions of *gyrA* and *parC* that we report have been previously described (49).

Although ciprofloxacin was discontinued more than a decade ago resistance rates remain high (47). Possibly explanations for this are the use ciprofloxacin in management of dysuria in men and as, in our experience, continued use of ciprofloxacin for treatment of male urethral discharge by some of the private practitioners in our area. The continuous high resistance rate means that repurposing of this drug for syndromic management is not feasible at this stage. The high rate of tetracycline resistance that we observed could be attributed to the use of doxycycline in syndromic management of non-gonococcal urethritis until five years ago in South Africa (10). Most of the tetracycline-resistant isolates harboured the well-known *tetM* plasmid and RpsJ V57M mutations (50). In addition, most of the isolates (70%) harboured both the GGI and plasmid mediated AMR. The GGI, a type 4 secretion system is associated with the spread of AMR among the gonococcal populations to multiple antimicrobials (39). The high prevalence of the GGI in our study is in line with a study in Kenya where 94% gonococcal strains harboured both GGI and plasmid mediated AMR (6).Azithromycin was only introduced in 2015 in the syndromic management of urethral discharge in South Africa; prior to that it was not widely available for other indications in the public health sector (10). Despite the short period of time that azithromycin has been used to treat STIs, we already observed a 15% resistance in azithromycin among our isolates; all of these had been obtained from MSM. Moreover, a substantial number (22%) of *N. gonorrhoeae* strains had elevated MICs (0.5-0.75 mg/L) with an Ala-39→Thr mutation which would previously have been classified ‘resistant’ using the EUCAST breakpoint. However, EUCAST does no longer have azithromycin susceptibility breakpoint because azithromycin is always used in conjunction with another drug for treatment, instead ECOFFs are used to describe azithromycin resistance. This highlights

the importance of including male groups, and especially MSM, in routine drug surveillance. At this stage it is unclear to what extent azithromycin resistance is restricted to core group populations or whether it is extended across the general population in South Africa. National surveillance among symptomatic individuals at sentinel facilities suggest that there is still a low prevalence (<3%) of azithromycin intermediate resistance of *N. gonorrhoeae*, however, core transmission groups are not specifically included (47). On the other hand, recent data from two clinics in the Kwazulu-Natal province suggest the opposite as they detected azithromycin resistance in 68% of isolates (48). This study used agar dilution methods for MIC determination rather than E-test. Nevertheless, the three studies provide a clear signal that intensified monitoring of azithromycin resistance is highly warranted as emerging resistance may undermine the effectiveness of syndromic management. The UK STI treatment guidelines have abandoned the principle of dual therapy for gonorrhea, recommending ceftriaxone as first line treatment when antimicrobial susceptibility is unknown or ciprofloxacin only when the antimicrobial susceptibility to this drug is confirmed prior to treatment (51). Nonetheless, dual therapy of azithromycin and ceftriaxone will remain in effect in settings of syndromic management. Good news in that regard is that we did not detect any resistance for the cephalosporins or spectinomycin.

Genomic analysis of *N. gonorrhoeae* strains obtained in our relatively small group of participants shows a genetically diverse bacterial population. We identified a substantial number of novel NG-STAR and NG-MAST STs that have not been reported from elsewhere in the world. This emphasizes the importance of inclusion of local strain libraries and patients when considering new drugs or treatment regimens. Our study is one of the first studies in Africa to perform WGS of *N. gonorrhoeae* isolates and the first that successfully used culture-independent NG-MAST which could constitute an important low-cost alternative to WGS in resource-constraint settings. Our study demonstrates that genomic analysis of *N. gonorrhoeae*

strains circulating in core transmission groups can be achieved in a low-resource setting and that it may provide important additional insights in the occurrence and basis of antimicrobial resistance.

This study has several limitations, especially the relatively small sample size. For reasons of feasibility we aimed at recruiting high-risk men from core transmission groups, at which we succeeded. Although uptake was high, these groups are difficult to target in the South African public health setting. However, despite the low number of participants, this study provides an overview of the *N. gonorrhoeae* strains circulating in this population with a concerning message suggesting emerging drug resistance. Second, despite direct inoculation on agar plates, microbial culture remained negative in about one-third of participants with a positive molecular test for *N. gonorrhoeae*. This reflects the challenges of obtaining specimens in a clinic and transporting these to the laboratory in low-resource settings. Based on the overlap in NG-MAST STs we have no reason to assume that the uncultivated strains are different from those that were cultivated, however due to the small sample size this cannot be concluded.

Antimicrobial resistance in *N. gonorrhoeae* with the threat of untreatable gonorrhea is a global health concern. Our study provides a clear signal that a stronger focus is required on this infection in low-resource settings including the public healthcare sector in South Africa. We confirm the high resistance rates to ciprofloxacin and tetracycline which have been used for treatment of STIs for many years in South Africa. However, the emergence among MSM of azithromycin resistance only a few years after its introduction in syndromic management is highly concerning. There are no data on azithromycin resistance in this population prior to its introduction, however, national surveillance reported a prevalence of less than 3% (47). Many MSM in our setting have both male and female sex partners facilitating transmission from this relatively contained group to the general population. With an epidemic of MDR gonorrhea

looming it is imperative to strengthen sexual healthcare services in the country. Intensified clinical governance and antimicrobial stewardship, introduction of molecular diagnostics, careful selection of empirical treatment regimens, evaluation of new potential drugs such as zoliflodacin (52), and investment in an enhanced antimicrobial surveillance structure that includes core transmission groups is essential to pre-empt such development and to avoid an epidemic of untreatable gonorrhea in sub-Saharan Africa.

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All authors: none to declare.

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