Neisseria gonorrhoeae: testing, typing and treatment in an era of increased antimicrobial resistance

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CHAPTER 7

Decreased azithromycin susceptibility of *Neisseria gonorrhoeae* isolates in patients recently treated with azithromycin

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ABSTRACT

Background

Increasing azithromycin usage and resistance in *Neisseria gonorrhoeae* threatens current dual treatment. Because antimicrobial exposure influences resistance, we analysed the association between azithromycin exposure and decreased susceptibility of *N. gonorrhoeae*.

Methods

We included *N. gonorrhoeae* isolates of patients visiting the Amsterdam STI Clinic between 1999 and 2013 (t_0), with another visit in the previous 60 days (t_{-1}). Exposure was defined as the prescription of azithromycin at t_{-1}. We included one isolate per patient. Using multivariable linear regression we assessed the association between exposure and azithromycin minimum inhibitory concentration (MIC). Whole genome sequencing (WGS) was performed to produce a phylogeny, identify multilocus sequence types (MLST), multiantigen sequence types (NG-MAST), and molecular markers of azithromycin resistance.

Findings

We included 323 isolates: 212 were unexposed to azithromycin, 14 were exposed ≤30 days, and 97 were exposed between 31–60 days before isolation. Mean azithromycin MIC was 0.28 mg/L (range, <0.016–24 mg/L). Linear regression adjusted for age, ethnicity, infection site, and calendar year showed a significant association between azithromycin exposure ≤30 days and MIC (ß, 1.00; 95% CI, 0.44–1.56; P = 0.002). WGS was performed on 31 isolates: 14 unexposed, 14 exposed to azithromycin ≤30 days before isolation, and three t_{-1} isolates. Exposure to azithromycin was significantly associated with A39T or G45D *mtrR* mutations (P = 0.046), but not with MLST or NG-MAST molecular types.

Interpretation

The results suggest that frequent use of azithromycin in populations at high risk of contracting *N. gonorrhoeae* induces an increase in MIC, and may result in resistance.

INTRODUCTION

Antimicrobial resistance in *Neisseria gonorrhoeae* is a growing worldwide challenge. To restrain the development of resistance, international guidelines recommend dual therapy consisting of ceftriaxone plus azithromycin.[1,2] However, increasing resistance to azithromycin, an outbreak of high-level azithromycin resistant strains in the United Kingdom, and the first treatment failure of dual therapy could indicate an opposite effect.[3-5] Azithromycin has a considerable half-life of 2–4 days, resulting in sub-therapeutic plasma and tissue concentrations for up to 20 days.[6] If an infection with *N. gonorrhoeae* is acquired in this period, exposure to sub-therapeutic azithromycin concentrations and selection of resistant strains could occur. Azithromycin is often prescribed for sexually transmitted infections (STIs), e.g. for *Chlamydia trachomatis*, *N. gonorrhoeae*, and the syndromic management of urethritis.[1,7-10] In addition, it is widely used for respiratory tract and cutaneous infections, and for patients with penicillin allergy.[11,12] This results in high azithromycin exposure of populations at high risk of contracting gonorrhoea, such as men who have sex with men (MSM), or commercial sex workers. Therefore, we conducted a retrospective cohort study to determine if recent treatment with azithromycin was associated with decreased azithromycin susceptibility of *N. gonorrhoeae*.

METHODS

Study population

Patients who visited the STI Outpatient Clinic in Amsterdam, the Netherlands, between 1999 and 2013, were eligible for inclusion if they had a positive *N. gonorrhoeae* culture (t_0), and a previous clinic visit (t_{-1}) in the 60 days preceding gonorrhoea diagnosis. Exposure was defined as a prescription of azithromycin at t_{-1}. We excluded patients who reported antibiotic use other than those prescribed at the STI clinic, in the 3 months preceding gonorrhoea diagnosis. We included one episode and one isolate per patient. If a patient had both exposed and unexposed episodes, we included the exposed episode. In case of multiple exposed or multiple unexposed episodes, we included the most recent episode. In case of multiple *N. gonorrhoeae* isolates per episode (because of multiple infected anatomical sites), we included the isolate with the highest minimum inhibitory concentration (MIC) for azithromycin. Baseline and clinical characteristics were obtained from the electronic patient file. In this retrospective study, we used routinely acquired data and samples; therefore no informed consent was required.

Due to the half-life of azithromycin we considered not only exposure, but also time since exposure to be of possible influence. Therefore, the cohort of patients was divided into three exposure groups: those unexposed in the previous 60 days, those exposed in the previous 31–60 days, and those exposed in the previous 30 days.

Determination of azithromycin susceptibility

N. gonorrhoeae isolates were retrieved from storage, inoculated on chocolate agar, and incubated at 37°C in a 5% CO_2-enriched atmosphere. A 0.5 (0.45–0.55) McFarland standard solution of isolates in phosphate-buffered saline was inoculated on GC agar plates. Azithromycin MICs were determined using Etest as instructed by the manufacturer (bioMérieux SA, Marcy-l'Etoile, France).

Whole genome sequencing (WGS) and molecular typing

To determine the correlation of azithromycin exposure with sequence type (ST) and molecular resistance markers, we selected 31 isolates for WGS analysis. We selected all 14 isolates from patients exposed to azithromycin ≤30 days, and 14 unexposed isolates frequency matched for MIC and year of isolation. If exposed patients were also diagnosed with gonorrhoea at the t_{-1} visit, these t_{-1} isolates were selected as well.

The selected isolates were sent to the National Microbiology Laboratory of the Public Health Agency of Canada for WGS as previously described.[13,14] In brief, DNA samples were extracted from cultures following standard protocol using Epicentre Masterpure Complete DNA and RNA Extraction Kit (Mandel Scientific, Guelph, ON, Canada). Libraries were created with TruSeq sample preparation kits (Illumina, San Diego, CA, USA), and sequenced on the Illumina MiSeq platform (Illumina) yielding an average of 1,383,601 reads/genome and average genome coverage of 186X. Quality reads were assembled with SPAdes and annotated with Prokka. There was an average of 59.1 contigs per isolate and an average contig and N50 length of 37,728 and 87,813 nucleotides, respectively. A core single nucleotide variation (SNV) phylogeny was created, with the assembled contigs file of isolate 28 as a mapping reference and a custom Galaxy SNVPhyl workflow (https://github.com/phac-nml/snvphyl-galaxy). Highly recombinant regions with >10 SNVs per 100 nucleotides were removed from the analysis. A meta-alignment of informative core SNV positions was used to create a maximum likelihood phylogenetic tree, and phylogenetic clades were determined by cluster analysis using a genetic distance threshold of 4.5%. The percentage of valid and included positions in the core genome was 39.2% and 4,383 sites were used to generate

the phylogeny. WGS read data were submitted to the NCBI Short Read Archive under BioProject ID PRJNA348107.

N. gonorrhoeae multiantigen sequence types (NG-MAST), multilocus sequence types (MLST), and molecular markers associated with azithromycin resistance were determined *in silico*. Sequences were submitted to the NG-MAST website (www.ng-mast.net), and the MLST website (pubmlst.org/neisseria) to assign STs.[14,15] The selected resistance markers included: C2611T and A2059G mutation of the 23S rRNA gene (*Escherichia coli* numbering), presence of *erm*, *mtrR* –35A deletion, *mtrR* A39T, and *mtrR* G45D. Mutations of 23S rRNA were determined using the core SNV pipeline and *N. gonorrhoeae* NCCP11945 as a mapping reference.[13] *PenA* types were determined by submitting sequences to NG-STAR (ngstar.canada.ca).

Statistical analysis

Baseline characteristics, geometric mean azithromycin MICs, STs and molecular markers were compared using X^2, Fisher's exact or Kruskal–Wallis testing. Year of infection was used continuous, or categorized in 5-year groups (1999–2003, 2004–2008, and 2009–2013), if applicable. The association between azithromycin exposure and MIC of the isolates (transformed to its natural logarithm) was determined using multivariable linear regression analysis. The multivariable model included all variables with $P < 0.05$. In case of correlated variables (such as sexual risk group, anatomical site and human immunodeficiency virus [HIV] status), we included the variable of most clinical interest.

Given the half-life of azithromycin, the effect of sub-therapeutic azithromycin concentrations is expected to be most profound in the first month after treatment. Therefore, we performed a sub-analysis using multivariable linear regression restricted to patients with a t_{-1} visit in the 30 days before gonorrhoea diagnosis. All analyses were performed using Stata (version 13; StataCorp, College Station, TX, USA).

RESULTS

Included patients and *N. gonorrhoeae* isolates

At the Amsterdam STI clinic 340,592 consultations were recorded in 1999–2013. After applying the inclusion and exclusion criteria, 395 patients with one isolate were

included (Figure 1). Antimicrobial susceptibility could not be determined for 72 isolates, which were excluded. This resulted in 14 patients exposed to azithromycin ≤30 days, 97 patients exposed between 31–60 days, and 212 patients who were unexposed to azithromycin at t_{-1}.

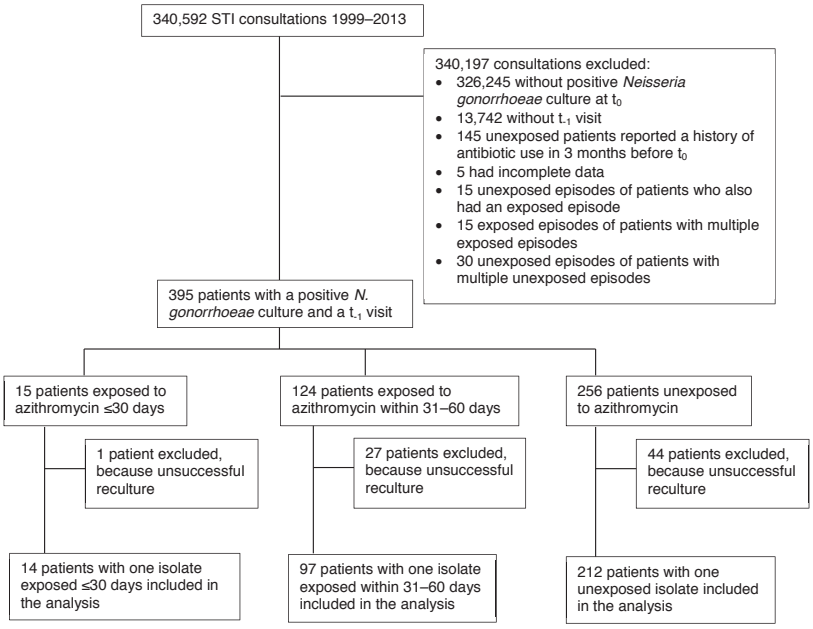


Figure 1. Flowchart of included patients

STI, sexually transmitted infection; t_0, visit of *N. gonorrhoeae* culture; t_{-1}, visit in 60 days preceding t_0 with (or without) azithromycin exposure.

Baseline characteristics

Patients were predominantly MSM (n = 235, 73%), and of Dutch ethnicity (n = 209, 65%). The median age was 33 years (interquartile range [IQR], 26–41 years). Two hundred thirty-one patients (72%) reported a previous gonorrhoea, and 209 (65%) reported a previous chlamydia diagnosis. The three exposure groups differed significantly for sexual risk group ($P = 0.02$), infection site ($P < 0.001$), HIV status ($P = 0.008$), and year of infection ($P < 0.001$; Table 1). Unexposed were more often MSM, with a rectal infection, HIV-positive and included recently (2009–2013), compared to those exposed. Among those exposed ≤30 days, four patients (29%) also had gonorrhoea at t_{-1}, compared to 51 (53%) patients exposed at 31–60 days, and 10 (5%) unexposed. All patients with gonorrhoea at t_{-1} were also treated with ceftriaxone or cefotaxime. There was no significant difference in MIC at t_0 between those with gonorrhoea at t_{-1} (mean, 0.28 mg/L; range, <0.016–12), and those without gonorrhoea at t_{-1} (mean, 0.28 mg/L; range, 0.032–24; $P = 0.7$).

Table 1. Baseline characteristics

Characteristics	Exposed ≤30 days (n = 14)[a]	Exposed 31–60 days (n = 97)[a]	Unexposed ≤60 days (n = 212)[a]	*P* value
Median age, years (IQR)	31 (27–32)	31 (24–38)	34 (26–41)	0.08
Ethnic group				0.2
Dutch	9 (64.3)	60 (61.9)	140 (66.0)	
Surinamese/Antillean	3 (21.4)	14 (14.4)	16 (7.6)	
Other	2 (14.3)	23 (23.7)	56 (26.4)	
Sexual risk group				0.02
Heterosexual male	4 (28.6)	23 (23.7)	23 (10.9)	
MSM	10 (71.4)	65 (67.0)	160 (75.5)	
Female	0 (0.0)	9 (9.3)	29 (13.7)	
Sex worker				0.2
No	14 (100.0)	93 (95.9)	191 (90.1)	
Yes	0 (0.0)	4 (4.1)	21 (9.9)	
Period of infection				<0.001
1999–2003	3 (21.4)	19 (19.6)	7 (3.3)	
2004–2008[b]	3 (21.4)	24 (24.7)	51 (24.1)	
2009–2013	8 (57.1)	54 (55.7)	154 (72.6)	
Site of infection[c]				<0.001
Tonsil	2 (14.3)	7 (7.2)	12 (5.7)	
Cervix	0 (0.0)	4 (4.1)	22 (10.4)	
Rectum	2 (14.3)	15 (15.5)	77 (36.3)	
Urethra	10 (71.4)	71 (73.2)	101 (47.6)	
Median time between t_{-1} and t_0, days (IQR)	27 (21–29)	48 (41–56)	42 (29–51)	<0.001
Gonorrhoea at t_{-1}	4 (28.6)	51 (52.6)	10 (4.7)	<0.001
Chlamydia trachomatis coinfection				0.7
No or not tested	12 (85.7)	80 (82.5)	167 (78.8)	
Yes	2 (14.3)	17 (17.5)	45 (21.2)	
HIV status				0.008
Negative	9 (64.3)	52 (53.6)	120 (56.6)	
Positive	3 (21.4)	30 (30.9)	83 (39.2)	
Missing	2 (14.3)	15 (15.5)	9 (4.3)	
Previous gonorrhoea episode[d]				0.002
No	3 (21.4)	15 (15.6)	73 (34.4)	
Yes	11 (78.6)	81 (84.4)	139 (65.6)	
Previous chlamydia episode[d]				0.1
No	2 (14.3)	30 (31.3)	81 (38.2)	
Yes	12 (85.7)	66 (68.8)	131 (61.8)	

IQR, interquartile range; MSM, men who have sex with men; HIV, human immunodeficiency virus; t_0, visit of *N. gonorrhoeae* culture, t_{-1}, visit in 60 days preceding t_0 with (or without) azithromycin exposure.
[a] Data are presented as No. (%) unless otherwise specified.
[b] No isolates from 2005 were included (lost from laboratory).
[c] Participants might be infected at multiple anatomical sites, but only one isolate per location was selected, i.e. the one with the highest azithromycin minimal inhibitory concentration.
[d] Ever reported gonorrhoea or chlamydia infection; 1 missing.

Azithromycin susceptibility

The geometric mean azithromycin MIC was 0.28 mg/L (range, <0.016–24), and differed significantly by sexual risk group (*P* <0.001), site of infection (*P* = 0.01), ethnicity (*P* <0.001), HIV status (*P* = 0.004), and time period of infection (*P* = 0.005; Table 2). The mean MIC was highest in isolates from MSM (0.34 mg/L; range, 0.023–24), in rectal infections (0.34 mg/L; range, 0.032–4), and in the most recent time period: 2009–2013 (0.30 mg/L; range, <0.016–24).

Table 2. Azithromycin MIC

	n (%)	MIC (range)[b]	*P* value
All patients[a]	323	0.28 (<0.016–24)	
Ethnic group			<0.001
Dutch	209 (64.7)	0.31 (<0.016–24)	
Surinamese/Antillean	33 (10.2)	0.13 (<0.016–1)	
Other	81 (25.1)	0.30 (0.023–12)	
Sexual risk group			<0.001
Heterosexual male	50 (15.5)	0.16 (<0.016–3)	
MSM	235 (72.8)	0.34 (0.023–24)	
Female	38 (11.8)	0.18 (<0.016–8)	
Site of infection			0.01
Tonsil	21 (6.5)	0.19 (0.023–0.75)	
Cervix	26 (8.1)	0.20 (0.023–8)	
Rectum	94 (29.1)	0.34 (0.032–4)	
Urethra	182 (56.4)	0.28 (<0.016–24)	
Period of infection			0.005
1999–2003	29 (9.0)	0.19 (0.023–12)	
2004–2008	78 (24.2)	0.26 (<0.016–4)	
2009–2013	216 (66.9)	0.30 (<0.016–24)	
HIV status			0.004
Negative	181 (56.0)	0.25 (<0.016–12)	
Positive	116 (35.9)	0.34 (0.032–24)	
Missing	26 (8.1)	0.23 (0.023–12)	
Gonorrhoea at t_{-1}			0.73
No	258 (79.9)	0.28 (<0.016–12)	
Yes	65 (20.1)	0.28 (0.032–24)	
Azithromycin at t_{-1}			0.1
Unexposed ≤60 days	212 (65.6)	0.28 (<0.016–12)	
Exposed 31–60 days	97 (30.0)	0.25 (0.023–12)	
Exposed ≤30 days	14 (4.3)	0.56 (0.19–24)	

MIC, minimum inhibitory concentration in mg/L; MSM, men who have sex with men; HIV, human immunodeficiency virus; t_0, visit of *N. gonorrhoeae* culture, t_{-1}, visit in 60 days preceding t_0 with (or without) azithromycin exposure.

[a] In case of multiple isolates per patient, only the isolate with the highest MIC for azithromycin was included.

[b] Geometric mean MIC (range).

Determinants of azithromycin MIC

Figure 2 shows the azithromycin MIC by time since t_{-1}, for each exposure group. Univariable linear regression showed a significant association between azithromycin MIC and exposure group (P = 0.03), age (P <0.001), ethnicity (P <0.001), sexual risk group (P <0.001), year of infection (P = 0.03), anatomical site of infection (P = 0.04), and HIV status (P = 0.03; Table 3). Anatomical site of infection and sexual risk group were correlated (only women provided cervical samples), as were HIV-positivity and sexual risk group (all but one HIV-positive patients were MSM). Because anatomical site was of more clinical interest, only this was included in the multivariable analysis.

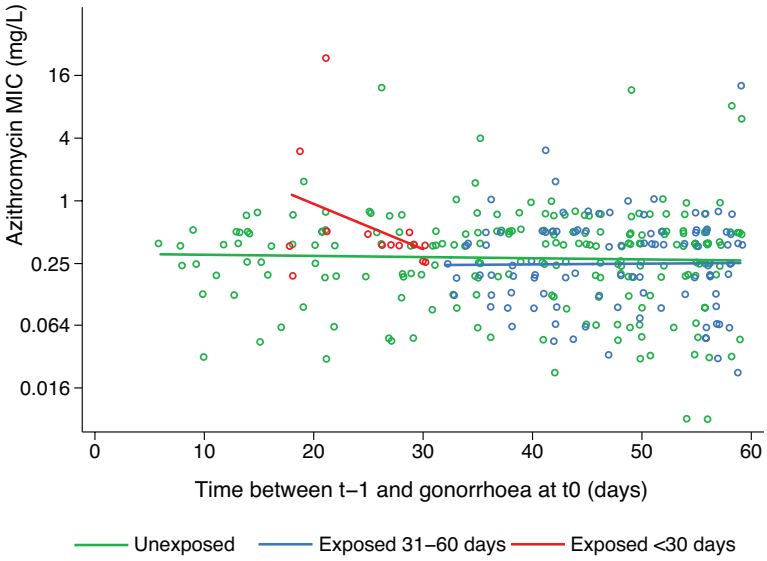


Figure 2. Scatterplot of azithromycin MIC by time since t_{-1}, according to exposure status

On the y-axis the azithromycin MIC is displayed on a natural logarithmic scale. The lines represent the linear function of MIC values in each exposure group.

MIC, minimum inhibitory concentration; t_0, visit of *N. gonorrhoeae* culture; t_{-1}, visit in 60 days preceding t_0 with (or without) azithromycin exposure

When adjusting for age, ethnicity, anatomical site and year of infection, we found a significant association between exposure group and azithromycin MIC (P = 0.002). Patients exposed to azithromycin ≤30 days had isolates with significantly higher azithromycin MICs (ß, 1.00; 95% confidence interval [CI], 0.44–1.56; P = 0.001) compared to unexposed. This means that the mean MIC of isolates from patients exposed ≤30 days was 2.7 times higher than that of unexposed. The mean MIC from isolates of patients exposed at 31–60 days was not significantly different compared to unexposed (ß, 0.07; 95% CI, -0.19 to 0.33; P = 0.6).

We noted a possible outlier among the exposed ≤30 days group (MIC, 24 mg/L; Figure 2). When excluding this outlier, the association between exposure ≤30 days and azithromycin MIC remained significant (ß, 0.75; 95% CI, 0.17–1.32; *P* = 0.04).

We performed the same multivariable analysis, but now adjusting for sexual risk group instead of anatomical site; the association between exposure ≤30 days and MIC was significant (ß, 0.95; 95% CI, 0.40–1.51; *P* = 0.003), also when excluding the outlier (ß, 0.70; 95% CI, 0.13–1.27; *P* = 0.049).

Sub-group analyses

A sub-analysis restricted to patients with a t_{-1} visit ≤30 days preceding t_0 (n = 71) showed a significant association between azithromycin exposure and MIC (ß, 1.08; 95% CI, 0.43–1.74; *P* <0.001). This effect remained significant when excluding the possible outlier (ß, 0.76; 95% CI, 0.12–1.40; *P* = 0.01).

Because those exposed ≤30 days did not include any women, we performed a sub-analysis restricted to men (n = 285). The association between exposure to azithromycin ≤30 days and MIC was significant (ß, 0.98; 95% CI, 0.44–1.52; *P* = 0.002), also when excluding the outlier (ß, 0.72; 95% CI, 0.17–1.27; *P* = 0.04).

Phylogenomic analysis

We selected 31 isolates for WGS: 14 from patients exposed ≤30 days, 14 unexposed, and three t_{-1} isolates. One other t_{-1} isolates was non-viable. The 31 isolates were obtained from 28 patients: 20 MSM (71%), 5 heterosexual males (18%), and 3 females (11%), and the majority was Dutch (n = 19; 68%; Figure 3). Isolates were mainly collected in 2009–2013 (n = 19; 61%). Six isolates were resistant to azithromycin (MIC >1 mg/L). All were susceptible to ceftriaxone (MIC <0.125 mg/L), and one was intermediate for cefixime (MIC = 0.094 mg/L).

Table 3. Determinants of azithromycin MIC, results of linear regression analysis[a]

		Univariable			Multivariable		
	n (%)[b]	ß	95% CI	*P* value	ß	95% CI	*P* value
Age, per year (median [IQR])	33 (26–41)	0.03	0.02 to 0.04	<0.001	0.03	0.01 to 0.04	<0.001
Ethnic group				<0.001			<0.001
Dutch	209 (64.7)	0.84	0.45 to 1.23		0.68	0.29 to 1.07	
Surinamese/Antillean	33 (10.2)	Ref			Ref		
Other	81 (25.1)	0.81	0.38 to 1.24		0.80	0.38 to 1.21	
Sexual risk group				<0.001			
Heterosexual male	50 (15.5)	Ref					
MSM	235 (72.8)	0.78	0.46 to 1.10				
Female	38 (11.8)	0.17	-0.27 to 0.61				
Year of infection (median [range])[c]	2010 (1999–2013)	0.04	0.005 to 0.07	0.03	0.03	-0.004 to 0.06	0.08
Infection site				0.04			0.24
Tonsil	21 (6.5)	Ref			Ref		
Cervix	26 (8.1)	0.08	-0.54 to 0.71		0.35	-0.24 to 0.95	
Rectum	94 (29.1)	0.60	0.09 to 1.11		0.49	-0.001 to 0.98	
Urethra	182 (56.4)	0.38	-0.10 to 0.87		0.33	-0.13 to 0.80	
Azithromycin at t_{-1}				0.03			0.002
Unexposed ≤60 days	212 (65.6)	Ref			Ref		
Exposed 31–60 days	97 (30.0)	-0.13	-0.39 to 0.13		0.07	-0.19 to 0.33	
Exposed ≤30 days	14 (4.3)	0.69	0.11 to 1.28		1.00	0.44 to 1.56	
Chlamydia trachomatis coinfection				0.23			
No (or unknown)	259 (80.2)	Ref					
Yes	64 (19.8)	-0.18	-0.48 to 0.12				
HIV status				0.03			
Negative	181 (56.0)	Ref					
Positive	116 (35.9)	0.32	0.06 to 0.57				
Missing	26 (8.1)	-0.09	-0.53 to 0.36				
Previous gonorrhoea episode[d]				0.07			
No	91 (28.3)	Ref					
Yes	231 (71.7)	0.25	-0.02 to 0.51				
Previous chlamydia episode[d]				0.95			
No	113 (35.1)	Ref					
Yes	209 (64.9)	-0.01	-0.26 to 0.24				

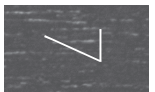
MIC, minimum inhibitory concentration; ß, regression coefficient for the natural logarithm of the azithromycin MIC; IQR, interquartile range; MSM, men who have sex with men; HIV, human immunodeficiency virus; t_0, visit of *N. gonorrhoeae* culture; t_{-1}, visit in 60 days preceding t_0 with (or without) azithromycin exposure.

[a] In case of multiple *N. gonorrhoeae* isolates per patient, only the isolate with the highest azithromycin MIC was included.

[b] unless otherwise specified.

[c] Per year of infection; no isolates from 2005 were included (lost in laboratory).

[d] Ever reported gonorrhoea or chlamydia infection; 1 missing.



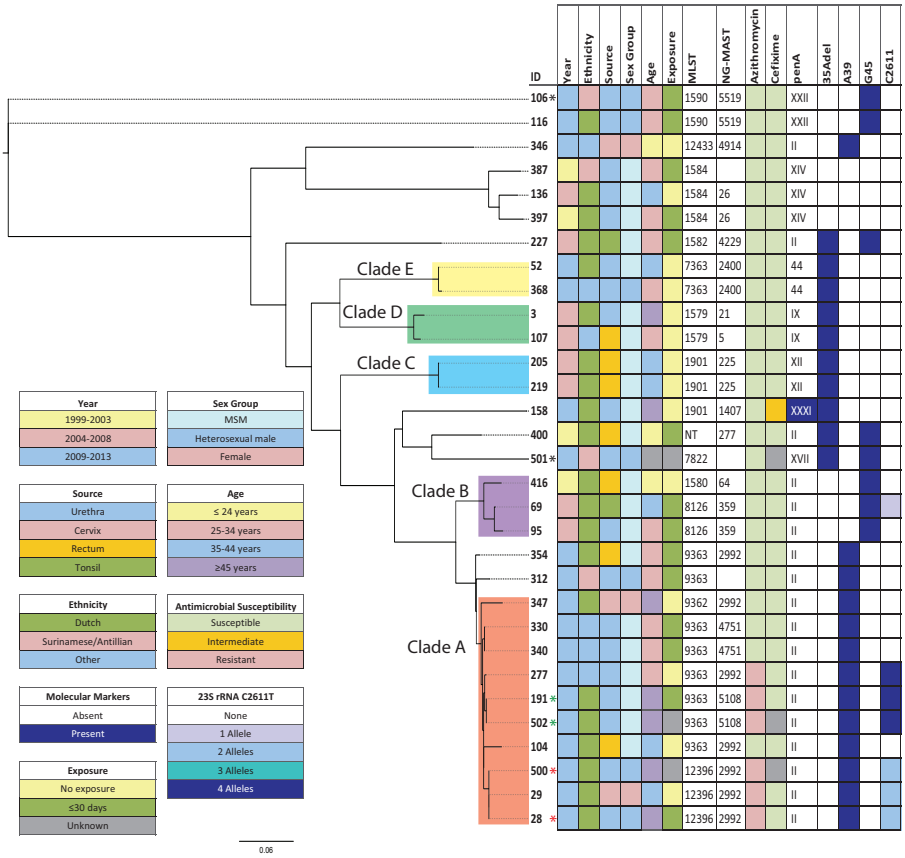


Figure 3. Whole genome core SNV maximum likelihood phylogenetic tree of 31 *N. gonorrhoeae* isolates

The length of the scale bar represents the estimated evolutionary divergence between isolates based on the average genetic distance between strains (estimated substitutions in sample/total high quality SNVs). All isolates were susceptible t_0 ceftriaxone and isolate ID 347 had *ermB*. Samples marked with coloured asterisks indicate a t_{-1} and t_0 pair of isolates from the same patient.

Heatmap columns: Year, year of isolate collection; Ethnicity, cultural background of patient; Source, anatomical site of isolate; Sex group, sex and sexual behaviour of patient; Age, age group of patient; Exposure, exposure and time since exposure to azithromycin; MLST, multilocus sequence type; NG-MAST, multiantigen sequence type, the sequence type is left blank for three isolates, as these await the assignment of a new sequence type; Azithromycin, susceptibility to azithromycin, with green as susceptible (MIC ≤0.5 mg/L), and red as resistant (MIC >1 mg/L); Cefixime, susceptibility to cefixime, with green as susceptible (MIC ≤0.063 mg/L), and orange as intermediate resistance (MIC >0.063 mg/L); *penA*, penicillin binding protein *penA* type, with blue indicating a mosaic allele; 35Adel, the –35A deletion in the *mtrR* promoter; A39, presence of A39T mutations in *mtrR*; G45, the presence of G45D mutations in *mtrR*; C2611, the number of C2611T mutated 23S rRNA alleles (using *E. coli* numbering).

MSM, men who have sex with men; NT, non typeable; SNV, single nucleotide variation; t_0, visit of *N. gonorrhoeae* culture; t_{-1}, visit in 60 days preceding t_0 with (or without) azithromycin exposure.

We identified 15 different NG-MAST STs, of which ST2992 was most common (n = 7; 23%), all other types were found in one or two strains. Three isolates had sequences which were not reported before, these await the assignment of a new ST number. We identified 10 different MLST types, of which ST9363 was most common (n = 8; 26%), followed by ST1901, ST1584, and ST12396 (n = 3; 10% each), one could not be typed due to a missing *pdhC* gene. The most common *penA* type was II (n = 18; 58%), and one (3%) was a mosaic *penA* type XXXIV.

We noted no A2059G mutations; C2611T mutations of 23S rRNA were observed in seven isolates (23%), consisting of 1 mutated allele (n = 1), 3 alleles (n = 3), or 4 alleles (n = 3). All isolates with at least 3 mutated alleles were resistant to azithromycin. The isolate with 1 mutated allele had an MIC of 0.38 mg/L.

An *ermB* gene was present in one isolate (3%), which had a wild-type 23S rRNA, an A39T *mtrR* mutation, and azithromycin MIC of 0.5 mg/L. Mutations in *mtrR* were noted in 28 isolates; 10 (36%) had a –35A deletion, 13 (46%) had an A39T mutation, and eight (29%) had a G45D mutation. Among isolates with an A39T or G45D mutation, the geometric mean azithromycin MIC was 0.84 mg/L, compared to 0.35 mg/L for isolates without these mutations (P = 0.08). A39T or G45D mutations were significantly more often seen among isolates from patients exposed ≤30 days (n = 12; 86%), compared with unexposed isolates (n = 6; 43%; P = 0.046). No significant associations between exposure to azithromycin and 23S rRNA mutations were observed.

WGS analysis grouped 19 isolates into five clades (clades A–E; Figure 3), and 12 isolates were outside these lineages. The largest clade (clade A; n = 10) consisted of MLST ST9362, ST9363, ST12396, and NG-MAST ST2992, ST5108, and ST4751; and contained only recent isolates collected from 2009–2013. Phylogenetic clustering of molecular markers was seen for *penA* type II isolates, which were predominantly located in clades A–B, while other *penA* types grouped into other clades and subclades. The *mtrR* –35A deletion was predominantly seen in isolates of clades C–E, whereas the A39T and G45D mutations were found in isolates of clades A and B, respectively. All azithromycin resistant isolates were located in clade A, and possessed a C2611T mutation in at least 3 alleles of 23S rRNA. Azithromycin exposure status did not cluster, and was distributed throughout the phylogeny; when comparing exposed ≤30 days to unexposed, no significant differences of NG-MAST (P = 0.12), MLST (P = 0.08), or *penA* types (P = 0.07) were observed.

The three pairs of patients exposed to azithromycin ≤30 days and with gonorrhoea at t_{-1}, had both t_{-1} and t_0 isolates tested with WGS. The first pair of isolates (501 and 106) were phylogenetically distant with different molecular profiles, indicating that the second infection was with a different strain. The second (502 and 191) and third pair (500 and 28) clustered closely; each pair had identical molecular profiles, suggesting a reinfection with the same strain, probably from an untreated sex partner. The paired isolates 500 and 28 (from a heterosexual male) were identical to isolate 29 (from a female commercial sex worker), and clinic visits of these patients were one week apart. However, we could not establish a partner link to confirm sexual contact between these patients.

DISCUSSION

We describe an association between exposure to azithromycin and decreased azithromycin susceptibility of *N. gonorrhoeae* in humans. Azithromycin MICs were significantly higher in isolates from patients exposed to azithromycin ≤30 days before gonorrhoea diagnosis, compared to patients treated 31–60 days previously, and to those not treated with azithromycin. There have been only two previous case reports that described an increase in azithromycin MIC after treatment with azithromycin, while MICs for other antimicrobials remained at pretreatment levels.[16,17] In other studies, azithromycin susceptible isolates were exposed to different concentrations of erythromycin *in vitro*, after which high-level resistance associated with 23S rRNA mutations developed rapidly.[18,19] Furthermore, pharyngeal macrolide-resistant viridans streptococci were more often found after treatment with macrolides, including azithromycin.[20] It is suggested that exposure of *N. gonorrhoeae* to low azithromycin concentrations can induce an increase in MICs, as was demonstrated for erythromycin, or cause selection of strains with an increased MIC.[4,6,19,21,22]

Although we were unable to evaluate molecular resistance markers for all isolates, WGS was performed to compare a subset of exposed and unexposed isolates. Phylogenetic analysis did not indicate any genetic relatedness based on azithromycin exposure. In addition, azithromycin exposure was not significantly associated with 23S rRNA mutations, suggesting that 23S rRNA mutations arose independently of exposure and spread clonally. However, A39T or G45D mutations of *mtrR* were significantly more common among isolates from patients exposed to azithromycin. This suggests that

sub-therapeutic levels of azithromycin might induce, or select for strains with these mutations. Our results indicate that higher MICs in strains from patients exposed to azithromycin were not caused by selection of a specific WGS clade, or MLST or NG-MAST type, but that acquisition, or selection of specific mutations, possibly including *mtrR*, is responsible for this effect. Future studies should be conducted to confirm our results, and identify what effect azithromycin treatment has on molecular resistance determinants in *N. gonorrhoeae*.

The current study has potential limitations. Sixty-five patients (20%) also had gonorrhoea at t_{-1}, and if treatment at t_{-1} failed due to azithromycin resistance, this may have caused selection bias. Because all were treated with cephalosporins, and mean MICs at t_0 were not different, we considered this highly unlikely. To prevent recall bias or incorrect classification of exposure, we only included azithromycin prescribed by the STI clinic, and excluded those reporting a recent history of using antibiotics prescribed elsewhere. Therefore, the influence of azithromycin exposure from other sources (general practitioners or medical specialists) has not been taken into account. Furthermore, there was one influential MIC outlier, but the association remained significant when excluding this outlier. Finally, the sample size of isolates from patients exposed to azithromycin in the previous 30 days was small. More isolates would be needed to find significant associations between exposure and molecular determinants of higher MICs.

Azithromycin is widely administered for *C. trachomatis* and non-specific urethritis in patients at high risk for STIs.[1,7-9] Moreover, most guidelines recommend dual therapy comprising azithromycin for the treatment of gonorrhoea.[1,2,10] In the light of emerging antimicrobial resistant *N. gonorrhoeae*, and lack of evidence-based alternative treatment options for multidrug resistant strains, prudence with antibiotics is warranted.[6] The current results add evidence to the possible negative effects of azithromycin on resistance formation in *N. gonorrhoeae*. As it is unclear whether dual therapy decreases selection pressure, and the first treatment failure of dual therapy is reported, the use of azithromycin in dual therapy for gonorrhoea, and in syndromic management of urethritis might need re-evaluation.[3,4,6] Moreover, research into new antimicrobial drugs, and the potential of currently available antibiotics for the treatment of gonorrhoea needs to be intensified. Future studies on the association between azithromycin exposure and resistance should focus on high-risk patients and molecular markers for resistance. They should include patients exposed to

azithromycin in the previous month, and azithromycin prescribed by other health care institutions than STI clinics.

In conclusion, our results show a significant association between exposure to azithromycin and higher azithromycin MICs in *N. gonorrhoeae*, without the selection of specific resistant genotypes. These results suggest that frequent use of azithromycin in patients at high risk of contracting *N. gonorrhoeae* infections induces an increase in MIC, possibly caused by mutations of *mtrR*, which may result in clinical resistance.

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