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High-Level Azithromycin Resistance Occurs in *Neisseria gonorrhoeae* as a Result of a Single Point Mutation in the 23S rRNA Genes▽

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High-level azithromycin resistance (AZM-HR), defined as a MIC of ≥256 mg/liter, emerged in *Neisseria gonorrhoeae* in the United Kingdom in 2004. To determine the mechanism of this novel phenotype, isolates from the United Kingdom that were AZM-HR (*n*, 19), moderately AZM resistant (MICs, 2 to 8 mg/liter) (*n*, 26), or sensitive (MICs, 0.12 to 0.25 mg/liter) (*n*, 4) were screened for methylase (*erm*) genes and for mutations in the *mtrR* promoter region, associated with efflux pump upregulation. All AZM-resistant isolates and 12 sensitive isolates were screened for mutations in domain V of each 23S rRNA allele. All AZM-HR isolates contained the A2059G mutation (*Escherichia coli* numbering) in three (3 isolates) or four (16 isolates) 23S rRNA alleles. Most (22/26) moderately AZM resistant isolates contained the C2611T mutation in at least 3/4 alleles. The remainder contained four wild-type alleles, as did 8/12 sensitive isolates, while one allele was mutated in the remaining four sensitive isolates. Serial passage of AZM-sensitive colonies on an erythromycin-containing medium selected AZM-HR if the parent strain already contained mutation A2059G in one 23S rRNA allele. The resultant AZM-HR strains contained four mutated alleles. Eight isolates (five moderately AZM resistant and three AZM-HR) contained mutations in the *mtrR* promoter. No methylase genes were detected. This is the first evidence that AZM-HR in gonococci may result from a single point mutation (A2059G) in the peptidyltransferase loop in domain V of the 23S rRNA gene. Mutation of a single allele is insufficient to confer AZM-HR, but AZM-HR can develop under selection pressure. The description of a novel resistance mechanism will aid in screening for the AZM-HR phenotype.

High-level resistance to azithromycin (AZM-HR) in *Neisseria gonorrhoeae* was documented for an isolate recovered in Argentina (6) in 2001 and is thought to have first emerged in Europe in Scotland in 2004. Subsequently, AZM-HR emerged in England and Wales (3, 14) and in Italy (18) in 2007.

Azithromycin (AZM) is not currently recommended as a treatment for gonorrhea in the United Kingdom, due to the requirement for higher doses (2 g), which are less well tolerated by patients (2), although this dosage is recommended as a treatment option for patients with penicillin hypersensitivity in the United States (12). However, AZM may be of greater therapeutic relevance either singly or as part of combination therapy in the future as the gonococcal population becomes less susceptible to the currently recommended expanded-spectrum cephalosporins, and treatment failures become increasingly common. The mechanism for the AZM-HR phenotype is currently unknown, but defining it would facilitate the development of screening tools that could be used in future patient management if nucleic acid amplification tests replace culture as a routine method of diagnosing gonorrhea.

AZM belongs to the macrolide-lincosamide-streptogramin (MLS) class of antibiotics and exerts its antimicrobial effect by binding to the 23S rRNA component of the 50S ribosome and inhibiting protein synthesis. Evidence suggests that low-level resistance to AZM in *N. gonorrhoeae* (MICs ranging from 1 to 4 mg/liter) can arise by a variety of mechanisms. Alteration of the drug target, 23S rRNA, either by mutation or by enzymatic modification is reported to contribute to low-level resistance. Genes (*ermB* and *ermF*) encoding methylase enzymes, which modify the 23S rRNA to prevent drug binding, have been identified in gonococcal isolates with low-level macrolide resistance (4, 15), and a 23S rRNA mutation (C2611T) has been proposed to contribute to the low-level resistance observed in two Canadian gonococcal strains (13). Efflux pumps, which actively expel agents to prevent sufficient intracellular accumulation for an antibacterial effect, are also well documented as playing a role in gonococcal macrolide resistance. The best characterized is the MtrCDE efflux pump, regulated by the MtrR repressor protein. Various mutations either in the *mtrR* gene or in the promoter region have been reported in gonococcal strains (4, 22), leading to low-level macrolide resistance as a result of decreased MtrR expression and upregulation of the MtrCDE efflux pump. However, other efflux pumps are also documented in *N. gonorrhoeae*, including the MacA-MacB system (16) and that encoded by the *mef* gene (10). Recently, one other study examining high-level AZM resistance in an Argentinean gonococcal strain demonstrated that the phenotype was not due to *mtrR* mutations or the presence of *mef* genes (6).

This study investigated the mechanism for the AZM-HR phenotype in *N. gonorrhoeae* and demonstrated for the first time that this phenotype arises due to point mutations in the peptidyltransferase loop of the 23S rRNA gene.

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TABLE 1. Sequence of the peptidyltransferase region of domain V of each 23S rRNA allele in 57 gonococcal isolates representing different AZM resistotypes and NG-MAST sequence types

Susceptibility to AZM (MIC range)	23S rRNA domain V allele sequence				NG MAST sequence type(s)[a]	No. of isolates
	Allele 1	Allele 2	Allele 3	Allele 4		
Sensitive (0.06–0.125 mg/liter)	WT[b]	WT	WT	WT	51, 87, 384, **649**, 1182, 2208, 3373, 3375	8
	WT	WT	WT	A2059G	**1443**, **1704**	2
	WT	A2059G	WT	WT	**470**	1
	WT	WT	C2611T[c]	WT	25	1
Moderately resistant (2–8 mg/liter)	WT	WT	WT	WT	2, 359	4
	C2611T	C2611T	C2611T	C2611T	359, 1195, 2322, 3150, 2597, 4236	19
	C2611T	C2611T	WT	C2611T	1195, 3150	3
Highly resistant (≥256 mg/liter)	A2059G	A2059G	A2059G	A2059G	470, 649, 1443, 1704, 2152, 2193	16
	A2059G	WT	A2059G	A2059G	1443	1
	A2059G	A2059G	WT	A2059G	225, 3311	2

[a] High-level AZM resistance has been documented for sequence types in boldface.
[b] WT, wild-type sequence.
[c] Only a single strand of sequence was of sufficient quality to determine mutations at position 2611.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. A total of 57 isolates of *N. gonorrhoeae* recovered from patients attending either Sexually Transmitted Infection (STI) clinics (*n*, 53), primary care (*n*, 3), or a hospital setting (*n*, 1) as part of their routine investigation and management were included in this study. All isolates had previously been typed by *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST), which examines sequence variation in two hypervariable genes, *por* and *tbpB* (11). The study collection was selected on the basis of AZM susceptibility and sequence type (ST) and included 19 isolates exhibiting high-level AZM resistance as determined by Etest (3), of which 10 were selected from Scotland to represent all STs associated with this phenotype, and the remaining 9 comprise all examples of this phenotype observed to date in England (*n*, 7), Wales (*n*, 1), and Ireland (*n*, 1). Additionally, the collection included a cross-section of 26 moderately AZM resistant isolates selected to represent the range of lower MICs (2 to 8 mg/liter) observed to date in England and 12 sensitive isolates (AZM MICs, 0.06 to 0.25 mg/liter) selected from Scotland (*n*, 4) on the basis of the ST matching those of highly resistant strains and from regions of England (*n*, 8) where high-level resistance had been observed over the same period.

All isolates were retrieved from storage at −80°C in a cryopreservative by 18 to 20 h of incubation (36°C, 5% CO_2) on GC agar base (BD) containing 1% IsoVitaleX (GC VIT agar). The resultant colonies were subcultured, and DNA was prepared by boiling a suspension of an 18-h culture (equivalent to a McFarland standard of 1.0) for 10 min, centrifuging (10,000 rpm for 5 min), and retaining the supernatant for storage at −20°C until it was required for subsequent PCR assays.

PCR amplification and sequence-based investigation of AZM resistance mechanisms. To fully examine the role of mutations in the peptidyltransferase loop of domain V of the 23S rRNA gene, all 57 isolates were tested. Because *N. gonorrhoeae* contains four copies of this gene, all four alleles were amplified individually by PCR as described previously, using primers gonrRNA-F (5′-ACG AAT GGC GTA ACG ATG GCC ACA-3′) and one of four allele-specific reverse primers 23SrRNAR-allele 1 (5′-TCA GAA TGC CAC AGC TTA CAA ACT-3′), 23S rRNAR-allele 2 (5′-GCG ACC ATA CCA AAC ACC CAC AGG-3′), 23S rRNAR-allele 3 (5′-GAT CCC GTT GCA GTG AAG AAA GTC-3′), and 23S rRNAR-allele 4 (5′-AAC AGA CTT ACT ATC CCA TTC AGC-3′) (13). Domain V of the 23S rRNA gene of each allele was then sequenced in the forward and reverse directions using primers gonrRNA-F and gonrRNA-R (5′-TTC GTC CAC TCC GGT CCT CTC TCG TA-3′), described previously (13).

The *mtrR* promoter region was examined in all AZM-resistant isolates (*n*, 45) and the 4 sensitive isolates from Scotland by sequencing, as described previously (4), for mutations associated with upregulation of the MtrCDE efflux pump. These 49 isolates were also screened by PCR for production of the *ermA*, *ermB*, and *ermC* genes, encoding methylases, by using a previously described method (15).

In vitro selection of high-level AZM resistance. Bacterial suspensions of the four AZM-sensitive isolates from Scotland were prepared from single-colony expansions, and approximately 1×10^8 CFU, as determined by a viable count, was spread on GC agar (BD diagnostics, Oxford, United Kingdom) supplemented with 1% IsoVitaleX and containing a range of erythromycin (Sigma-Aldrich Company Ltd., Gillingham, United Kingdom) concentrations diluted 2-fold from 8.0 to 0.5 mg/liter. Plates were examined for bacterial growth after 24 h of incubation at 36°C under 5% CO_2. Individual colonies, where visible, were counted, and any visible growth on the plate containing the highest concentration of erythromycin was subcultured on GC VIT agar for species confirmation by Gram staining, an oxidase test, and the Phadebact Monoclonal GC test (Bactus AB, Huddinge, Sweden), performed according to the manufacturer's instructions. MICs of erythromycin and AZM were determined by Etest (bioMérieux UK Ltd., Basingstoke, United Kingdom) as described previously (3), and DNA lysates were prepared by boiling bacterial suspensions for 10 min. This experiment was repeated for isolates exhibiting higher erythromycin MICs than the parent strain, by using a higher range of concentrations of erythromycin, beginning at two times the MIC.

Transformation experiments. Transformation experiments were conducted as described previously (1). Briefly, 100-μl portions of suspensions (equivalent to a McFarland standard of 3.0) of piliated colonies of the laboratory-adapted strains FA19 and FA62 were each incubated for 6 h at 36°C under 5% CO_2 on the surface of a GC VIT agar plate with 100 μl of whole-DNA or 23S rRNA amplicons (712 bp) from AZM-HR strains. Bacterial growth was harvested and suspended in 1 ml peptone broth, and 100 μl of each suspension was then spread onto GC VIT agar plates containing erythromycin at concentrations ranging from 0.5 to 4.0 mg/liter. Plates were examined at 24-h intervals for as long as 5 days for evidence of bacterial transformants.

RESULTS

Characterization of putative resistance determinants. Sixteen of the 19 AZM-HR isolates tested (84%) contained mutation A2059G (*Escherichia coli* numbering) in all four alleles, while the remaining three isolates contained three mutated alleles and one wild-type allele (Table 1). One of the two isolates of sequence type 1443, with the AZM-HR phenotype, contained four mutated alleles, whereas the other isolate of this type contained three mutated alleles and one wild-type allele. None of the AZM-HR isolates contained the C2611T mutation reported previously (13).

In contrast, 75% (*n*, 9) of the 12 AZM-sensitive gonococcal isolates tested contained wild-type sequence in domain V of all four 23S rRNA alleles, while 3 of the 4 sensitive isolates of an NG-MAST sequence type where the AZM-HR phenotype has also been observed contained an A2059G mutation in one of

TABLE 2. Changes in the AZM MIC and domain V of each 23S rRNA allele following selection by erythromycin

Isolate no. (ST[a]) and stage of selection by erythromycin	AZM MIC (mg/liter)	23S rRNA domain V allele sequence			
		Allele 1	Allele 2	Allele 3	Allele 4
136 (ST 1704)					
Preselection	0.25	WT[b]	WT	WT	A2059G
Postselection	>256	A2059G	A2059G	A2059G	A2059G
352521 (ST 1443)					
Preselection	0.25	WT	WT	WT	A2059G
Postselection	>256	A2059G	A2059G	A2059G	A2059G
252611 (ST 470)					
Preselection	0.38	WT	A2059G	WT	WT
Postselection	>256	A2059G	A2059G	A2059G	A2059G
254921 (ST 649)					
Preselection	0.25	WT	WT	WT	WT
Postselection	1.5	WT	WT	WT	WT

[a] ST, NG-MAST sequence type.
[b] WT, wild type.

the four alleles (Table 1). All moderately resistant isolates lacked the A2059G mutation, but most (23/26) contained mutation C2611T in at least three 23S rRNA alleles (Table 1).

Examination of the promoter region for *mtrR* showed a wild-type sequence for all sensitive isolates tested (*n*, 4). The majority (21/26 [80.1%]) of the moderately resistant isolates were wild type, and the remaining five isolates contained single-base (A) deletions (*n*, 4) or single-base (T) insertions (*n*, 1) in the 13-bp inverted-repeat region of the *mtrR* promoter. Of the 19 AZM-HR isolates tested, 16 were wild type, and the remaining three isolates had a single-base (A) deletion in the 13-bp inverted repeat (*n*, 1) or a single-base (A) deletion 6 bp upstream of the −35 region of the promoter (*n*, 2).

Methylase genes (*ermA*, *ermB*, and *ermC*) were not detected by PCR in any of the isolates tested.

Transformation and *in vitro* selection of high-level AZM resistance. Experiments to transform erythromycin- and AZM-sensitive isolates with genomic DNA or 23S rRNA DNA amplicons from AZM-HR strains did not result in any transformants exhibiting high-level AZM resistance.

Serial passage of suspensions of expanded single-colony picks from four different gonococcal strains with erythromycin MICs of 0.5 to 0.75 mg/liter and azithromycin MICs of 0.25 to 0.38 mg/liter by Etest (Table 2) on media containing erythromycin concentrations ranging from 0.5 mg/liter to 8.0 mg/liter quickly selected for high-level resistance in 3/4 isolates, within one to two passages. For each of these three strains (strains 136, 352521, and 252611), each of which initially contained one mutated 23S rRNA allele (Table 2), ≥300 CFU was observed on the plates containing 8.0 mg/liter erythromycin. Subsequent testing of growth on this plate by Etest showed a population of mixed AZM susceptibilities for all three strains, with a highly resistant subpopulation (MIC, >256 mg/liter) in all cases. The purified highly AZM resistant population, examined further, contained four mutated 23S rRNA alleles in all three cases (Table 2).

The remaining strain (254921), with wild-type sequence in all four 23S rRNA alleles, failed to grow on plates containing ≥4.0 mg/liter erythromycin. Repeat passage of colonies observed on the 2.0-mg/liter medium onto a medium containing 2.0 mg/liter to 8.0 mg/liter raised the erythromycin MIC from 3.0 mg/liter to 4.0 mg/liter, as confirmed by Etest, but high-level erythromycin or AZM resistance did not develop. The 23S rRNA allele sequence remained wild type in the strain variant showing an increased erythromycin MIC of 4.0 mg/liter (AZM MIC, 1.5 mg/liter) (Table 2).

DISCUSSION

High-level AZM resistance in *Neisseria gonorrhoeae* is a relatively new phenomenon, and the mechanism for this novel phenotype remains unknown. This study presents the first evidence that high-level resistance to AZM occurs in *N. gonorrhoeae* as a result of a single point mutation in the peptidyltransferase region of domain V of the 23S rRNA gene.

AZM and other macrolides exert a bacteriostatic effect by interacting directly with the central loop of domain V, the site of peptide bond formation, thereby inhibiting protein synthesis. Mutations at various positions within this region are thought to confer resistance by reducing the ability of the drug to bind to its target. All AZM-HR isolates included in the current study contained mutation A2059G in at least three of the four alleles. It is well documented that mutations at position A2058 or A2059 confer the highest level of macrolide resistance on other bacterial pathogens, including *Helicobacter pylori* (20), *E. coli* (5), *Mycoplasma pneumoniae* (9), *Streptococcus pneumoniae* (19), and the genital pathogens *Treponema pallidum* (17) and *Mycoplasma genitalium* (8). While many of these pathogens contain just one or two copies of the *rrn* operon, and a mutation in one copy can be sufficient to confer resistance (20), *S. pneumoniae* is similar to *N. gonorrhoeae* in that it contains four copies. In that species it has been demonstrated that a point mutation (either A2058G or A2059G) in two of the four alleles is sufficient to confer high-level AZM resistance on laboratory mutants (19), and a Canadian survey of clinical pneumococcal isolates showed that isolates with one mutated allele could have relatively low erythromycin MICs (2 to 8 mg/liter) (21). In the current study, a single mutated allele

was insufficient to confer resistance on gonococcal isolates, presumably because the drug is still able to bind to the other three wild-type alleles. The effect of two mutated alleles is unknown, since no examples of this were observed in the current study. It is, however, evident that three mutated alleles will confer high-level resistance on gonococci.

The observation that all AZM-HR strains contained at least three mutated alleles, whereas a maximum of one mutated allele was observed in the sensitive and moderately resistant isolates, supports the role of the mutation A2059G in high-level resistance in gonococci. The C2611T mutation, reported previously in the 23S rRNA of moderately resistant gonococci (13), was not observed in any of the AZM-HR strains, indicating that this mutation does not contribute to this phenotype. In contrast, most (88%) of the moderately AZM resistant isolates contained this mutation in at least three alleles, providing supportive evidence for the role of mutation C2611T in lower-level resistance. Because mutations in the *mtrR* promoter were observed in isolates with lower-level resistance to AZM and were found in just 3 of the 19 AZM-HR strains in this study, it is unlikely that the MtrCDE efflux pump plays a significant role in the AZM-HR phenotype, although upregulation of this pump would provide resistance to hydrophobic molecules and other antibiotics, such as penicillin and tetracycline. Although *erm* genes were not detected in any of the AZM-resistant isolates, other mechanisms, which were not examined in the current study, such as mutations in the *mtrR* gene or the presence of *mef* genes, may also have contributed to the low-level resistance phenotype observed in the study collection. The PCR method published for testing for *ermF* (15) was also applied to the current study collection, and no amplicons were observed for any of the strains. However, since no appropriate positive control was available, the lack of *ermF* genes in the current study may be an artifact due to failure of the PCR assay.

AZM-sensitive isolates containing a single mutated 23S rRNA allele quickly developed high-level resistance in the presence of erythromycin. The domain V region of all four 23S rRNA alleles for each strain contained mutation A2059G, suggesting that endogenous homologous recombination may have occurred as a result of selection pressure. The rapid emergence of the AZM-HR phenotype was unlikely to be due to selection of a preexisting AZM-HR variant in the population, since the serial passage experiments were conducted in duplicate on cultures derived from a single colony. Furthermore, serial passage of a sensitive strain with fully wild type alleles did not result in high-level AZM resistance, indicating that at least one mutated allele is required for rapid development of this phenotype.

It was not possible to provide further supportive evidence of the role of mutation A2059G in the AZM-HR phenotype by transforming AZM-sensitive isolates to the resistant phenotype with DNA (whole-genome or 23S rRNA amplicons) from AZM-HR strains. It could be speculated that transformation to an AZM-HR phenotype possibly requires three simultaneous recombination events, and therefore our effort was unsuccessful, although other factors, such as insufficient colony piliation or lack of a proximal DNA uptake sequence, may have contributed to the failure as well. Alternatively, an as yet unidentified mutation present in the clinical isolates but absent in the recipient strains may be required, in addition to A2059G, to confer high-level resistance, although this is unlikely given the clear association between mutations in this region of the 23S rRNA gene and high-level macrolide resistance in other bacterial species. Because the acquisition of A2059G in a single allele would not result in AZM or erythromycin resistance, identification of transformants with a single mutated allele among the background population was not possible. No resistant colonies were observed following subculture of growth from plates containing low erythromycin concentrations onto higher concentrations, suggesting that if transformation had occurred, it was below the threshold for detection by this approach. Further work using alternative recipient strains may be warranted, but the current study nevertheless presents compelling evidence of the role of mutation A2059G in high-level AZM resistance in *N. gonorrhoeae*.

The observation that high-level AZM resistance develops rapidly in the laboratory in isolates with a single mutated allele is of concern, because it presents the possibility that high-level resistance could develop from an apparently sensitive isolate *in vivo* under a selection pressure. AZM is used extensively in the treatment of STIs other than gonorrhea in the United Kingdom and elsewhere. Patient treatment data collated for England and Wales as part of the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) indicate that patients with gonorrhea are increasingly prescribed AZM to treat *Chlamydia trachomatis* coinfection. Additionally, a minority of patients with gonorrhea did not receive specific antigonococcal therapy but were prescribed AZM only, in spite of their diagnosis (7). It is therefore evident that a selection pressure exists which could lead to the rapid emergence of highly resistant gonococci, particularly if specific treatment of gonococcal infection is inadequate. The distribution and prevalence of isolates with a single mutated allele in the gonococcal population in the United Kingdom or elsewhere are unknown, although this study has demonstrated that examples of this are already circulating in the gonococcal population in Scotland, among NG-MAST STs where the AZM-HR phenotype has also been observed. Future work should focus on the development of rapid molecular screening tools to evaluate this, as well as to detect AZM-HR strains. Based on the current study, we propose that AZM-HR gonococcal strains may have arisen in Scotland in a stepwise manner, first by the occurrence of mutation A2059G in a single allele, either spontaneously or by acquisition of exogenous mutated DNA from other bacterial species. High-level AZM resistance may have subsequently developed *in vivo* by internal recombination events under a selection pressure due to inadequate treatment of the gonococcal infection. Although an AZM-HR strain was first identified in Argentina in 2001, it had an NG-MAST type (ST 696) distinct from those observed in the United Kingdom, suggesting independent development of this phenotype (6).

The demonstration that high-level AZM resistance is likely to emerge rapidly in *N. gonorrhoeae* by a simple point mutation in the 23S rRNA highlights the importance of treating gonorrhea appropriately with the recommended therapies (expanded-spectrum cephalosporins in the United Kingdom and in many other countries worldwide). Inappropriate treatment of patients with suboptimal doses of AZM could lead to the selection and dissemination of the AZM-HR phenotype. Appropri-

ate antimicrobial prescribing combined with surveillance of AZM resistance in the gonococcal population will be critical in preventing this spread and therefore preserving future therapeutic options.

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