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Use of whole-genome sequencing data to analyze 23S rRNA-mediated azithromycin resistance

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Abstract

The whole-genome sequences of 24 isolates of *Neisseria gonorrhoeae* with elevated minimum inhibitory concentrations (MICs) to azithromycin (≥2.0 μg/mL) were analyzed against a modified sequence derived from the whole-genome sequence of *N. gonorrhoeae* FA1090 to determine, by signal ratio, the number of mutant copies of the 23S rRNA gene and the copy number effect on 50S ribosome-mediated azithromycin resistance. Isolates that were predicted to contain four mutated copies were accurately identified compared with the results of direct sequencing. Fewer than four mutated copies gave less accurate results but were consistent with elevated MICs.

Keywords

Azithromycin; Resistance; *Neisseria gonorrhoeae*; 23S rRNA

1. Introduction

In 2012, European sexually transmitted diseases guidelines began recommending the administration of ceftriaxone and azithromycin for gonococcal infections [1,2]. The use of dual-drug regimens reflects fears of untreatable gonorrhoea [3] and is predicated on the hypothesis that simultaneous emergence of resistance to each of these antibiotics will be

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2016.10.023.

infrequent and will be slowed by using them in combination [1]. However, strains resistant to azithromycin have been observed globally, with azithromycin minimum inhibitory concentrations (MICs) of ≥1.0 μg/mL associated with treatment failure [4,5]. The emergence of such strains highlights the need for tools to assist in the rapid identification of azithromycin-resistant isolates. Whole-genome sequencing (WGS) raises the possibility of developing molecular diagnostics to detect resistance and for use in ongoing surveillance and epidemiological monitoring of strain populations. Here we present an analysis of 23S rRNA-mediated resistance to azithromycin and compare the results from short-read WGS-based bioinformatic analysis with nested PCR and sequencing of the individual 23S rRNA copies.

Several genetic pathways appear to confer resistance to azithromycin. Mutations in *mtrR*, a transcriptional repressor that alters expression of the *mtrCDE* efflux pump operon, confer resistance to erythromycin and low-level resistance to azithromycin (0.5 μg/mL) [6,7]. These mutations include deletions and insertions within the inverted repeat in the *mtrR* promoter as well as point mutations within the structural gene [6]. In a set of clinical isolates from an outbreak in Kansas City (Missouri), a Correia element inserted into the *mtrCDE* promoter region between the transcriptional initiation site and the start codon of *mtrC* resulted in azithromycin MICs of ≥2.0 μg/mL. An additional insertion mutation in the 5′ coding region of the *mtrR* structural gene in a related cluster of strains increased the MIC to ≥4.0 μg/mL [8]. Mutational modifications in the 50S ribosomal target of azithromycin confer azithromycin resistance substantially greater than the resistance conferred by mutations in the *mtr*-mediated efflux system. The ribosomal mutations consist of characteristic mutations at one of two sites in the 23S rRNA, i.e. C2599T (*Neisseria gonorrhoeae* numbering) and A2059G (*N. gonorrhoeae* numbering)] [9–11]. *Neisseria gonorrhoeae* usually possesses four copies of the 23S rRNA per genome equivalent, and the greatest resistance has been reported for those isolates with all four copies as mutant alleles. In those instances where all four copies are mutant alleles C2599T confers moderate resistance (8–16 μg/mL), and the A2059G mutation confers high-level resistance (≥64 16 μg/mL) [10].

2. Materials and methods

2.1. Isolate selection and bioinformatic analysis

The ability of bioinformatic analysis of WGS data to accurately predict the number of mutant allele 23S rRNA copies in the genomes of *N. gonorrhoeae* isolates was investigated. Using a collection of gonococcal isolates with azithromycin MICs of ≥2.0 μg/mL taken from a previous larger study of 246 isolates for which full genome sequences and MIC data are available [12], the number of predicted 23S rRNA copies with the C2599T substitution was correlated with the azithromycin MIC. No substitutions at the 2059 site were observed in this collection. Isolates were selected, genomic DNA was extracted and sequenced, and the data were analyzed as described elsewhere [12]. The readings were mapped by SMALT [12] against a version of the *N. gonorrhoeae* FA1090 genome sequence (GenBank accession no. AE004969) from which three of the four 23S rRNA copies (those starting at nucleotides 116 247, 1 258 350 and 1 649 926) were deleted in silico. The ratio of C to T nucleotide calls from reads covering the 2599 site was determined by SAMtools [13] to establish the

predicted number of mutant allele copies present in a particular isolate. Based on the expectation of four genomic 23S rRNA copies in each isolate, the ratios were rounded to the nearest quartile.

2.2. Direct sequencing of 23S rRNA gene copies

Subsequently, the number of mutant allele copies present in each of the isolates was determined by directly sequencing each 23S rRNA copy in each isolate using the methods and primers described elsewhere [9]. Briefly, the copies were amplified separately using a common 23S rRNA primer with a unique external counter-primer adjacent to the desired copy. Each of these amplified fragments was then nested with internal rRNA primers that covered the region to be sequenced and included sites at which base substitutions responsible for azithromycin resistance are located.

3. Results and discussion

A total of 24 isolates with azithromycin MICs ≥ 2.0 μg/mL as determined by agar dilution were analyzed. Two isolates from the same collection with MICs of 1.0 μg/mL were included as sensitive controls and contained no mutations in any of the rRNA copies. The genomic sequencing results giving the estimated number of mutant alleles for each of the isolates were compared with the results obtained by directly sequencing each independent 23S rRNA copy (Table 1). Overall, isolates shown to have at least two mutant 23S rRNA copies by direct sequencing and an azithromycin MIC ≥ 2.0 μg/mL were correctly identified by WGS, indicating successful genotypic prediction of ribosome-mediated phenotypic resistance.

We further assessed the prediction of mutant copy number by WGS. Allowing for the omission of the single isolate that contained only three intact copies of the 23S rRNA (Table 1), the data showed that 18/19 (95%) of the isolates predicted by the WGS-based method to contain all copies as mutant alleles were confirmed by direct PCR and sequencing; only a single isolate was erroneously called by the WGS method. For estimated mutant copies less than four, the results were less accurate. Four isolates that were estimated by WGS methodology to contain two of four or three of four mutant copies agreed with the results from direct sequencing in only a single instance (Table 2). However, the extent of disagreement amounted to only a single copy for each discrepancy, and in no case of an azithromycin MIC ≥ 2.0 μg/mL was the WGS-based result less than two variant alleles. Of note, the use of a variant ratio dependent on the expectation of four genomic 23S rRNA copies does not allow for differences that may result from such issues as the atypical 23S rRNA copy numbers noted above. Within the relatively small collection of isolates analyzed here, one of them was found to possess three functional intact copies of the 23S rRNA, while the fourth copy appeared to have sustained a significant internal deletion that led to failed attempts at amplification. In addition, potential sources of error include polyploidy with 23S rRNA variation in genomes and undetected mixed populations.

The fraction of mutated 23S rRNA copies correlated with the MICs obtained. Those isolates with all 23S rRNA copies mutated gave azithromycin MICs (8.0 μg/mL and 16.0 μg/mL) higher than those with a fraction of the copies mutated, with the exception of a single isolate

(GCGC018) that despite possessing only three of four mutated of 23S rRNA gene copies exhibited an azithromycin MIC of 16.0 μg/mL. It is tempting to speculate that this isolate possesses at least one additional mutation elsewhere in the genome that contributes to this unexpectedly high MIC. The relatively low MICs obtained for those isolates that possessed at least one wild-type sensitive copy of the 23S rRNA suggested that the presence of even a single sensitive allele influenced the MIC more than the absolute number of resistant alleles. Moreover, the absence of isolates with only a single C2599T allele suggests that a single mutant 23S rRNA copy alone does not confer an MIC above the 2.0 μg/mL threshold used to designate isolates with reduced susceptibility.

4. Conclusion

These findings show that a bioinformatic analysis of short-read WGS data correctly predicted an association between at least two copies of the C2599T 23S rRNA allele and reduced susceptibility to azithromycin. While the approach does not perform perfectly when judged against the results obtained by sequencing the nested PCR products from the individual alleles, it none the less accurately predicts an azithromycin-resistant genotype and broadly indicates an associated phenotype. In addition, analysis of WGS data may indicate additional mechanisms of azithromycin resistance, as for GCGS018, which exhibits an unusually high azithromycin MIC of 16.0 μg/mL for an isolate with three mutant 23S rRNA copies. With the possible exception of GCGS018, no other isolates indicated mechanisms of azithromycin resistance other than mutations in the 23S rRNA, nor were any of the other known mechanisms for azithromycin resistance at 2.0 μg/mL found. Finally, these findings document the first observation of a gonococcal isolate with three, rather than four, genomic copies of the 23S rRNA, emphasizing the plasticity of the gonococcal genome. The genome reads are available in the SRA, NCBI BioProject PRJEB2999.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Estimated and actual 23S rRNA allele distributions of strains with azithromycin minimum inhibitory concentrations (MICs) of ≥2.0 μg/mL

Strain #	Genomic estimated 23S rRNA allele distribution	Actual sequence determined 23S rRNA allele distribution	MIC (μg/mL)
GCGS039	ND	0/4	1.0[a]
GCGS196	ND	0/4	1.0[a]
GCGS018	4/4	3/4	16.0
GCGS038	4/4	4/4	8.0
GCGS096	4/4	4/4	16.0
GCGS098	4/4	4/4	16.0
GCGS102	3/4	2/4	2.0
GCGS104	2/4	3/4	2.0
GCGS106	4/4	4/4	8.0
GCGS110	4/4	4/4	16.0
GCGS118	3/4	4/4	8.0
GCGS120	4/4	4/4	8.0
GCGS128	4/4	4/4	8.0
GCGS136	4/4	4/4	8.0
GCGS142	4/4	3/3[b]	8.0
GCGS156	4/4	4/4	16.0
GCGS174	4/4	4/4	16.0
GCGS176	4/4	4/4	16.0
GCGS198	4/4	4/4	16.0
GCGS202	4/4	4/4	8.0
GCGS204	4/4	4/4	8.0
GCGS218	4/4	4/4	16.0
GCGS224	4/4	4/4	8.0
GCGS226	4/4	4/4	16.0
GCGS228	2/4	2/4	2.0
GCGS230	4/4	4/4	8.0

[a] Sensitive controls.

[b] Allele 4 did not amplify.

Table 2

Distribution of mutant rRNA alleles.

Mutant/total allelic distribution	No. of estimated calls	No. of estimated calls confirmed by sequencing	Azithromycin MIC (μg/mL)
4/4	19[a]	18	8 (*n* = 9), 16 (*n* = 9)
3/4	2	0	16 (n = 1), 2 (n = 1)
2/4	2	1	2 (*n* = 2)

MIC, minimum inhibitory concentration.

[a]GCGS142 (MIC = 8.0 μg/mL) was omitted from this analysis since one allele could not be amplified or sequenced