Rifampicin Resistance Associated with \textit{rpoB} Mutations in \textit{Neisseria gonorrhoeae} Clinical Strains Isolated in Austria, 2016 to 2020

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\textbf{ABSTRACT} Due to increasing rates of antimicrobial resistance (AMR) in \textit{Neisseria gonorrhoeae}, alternative treatments should be considered. To assess rifampicin’s potential as a gonorrhea treatment, we used \textit{rpoB} mutations to estimate rifampicin resistance in Austrian \textit{N. gonorrhoeae} isolates. We found 30\% of resistant isolates clustering in three main phylogenomic branches. Rifampicin resistance was associated with resistance to other antibiotics. Therefore, rifampicin cannot be recommended as an alternative gonorrhea treatment in Austria, even in combination therapy.

\textbf{IMPORTANCE} Gonorrhea, caused by \textit{Neisseria gonorrhoeae}, is one of the most common bacterial sexually transmitted infections. It is treated with antibiotics; however, an increasing number of \textit{N. gonorrhoeae} strains are resistant to currently used treatments. In this study, we explored the potential of rifampicin, another antibiotic, as a treatment option for gonorrhea. However, around 30\% of Austrian \textit{N. gonorrhoeae} strains investigated were already resistant to rifampicin, which would limit its benefit as a gonorrhea treatment.

\textbf{KEYWORDS} \textit{Neisseria gonorrhoeae}, antibiotic resistance, rifampicin, whole-genome sequencing

Gonorrhea is the second most common bacterial sexually transmissible infection in the EU and worldwide (1). Gonorrhea is treated with antibiotics; however, \textit{Neisseria gonorrhoeae} has developed or acquired antimicrobial resistance (AMR) to all antimicrobials previously recommended for empirical treatment of gonorrhea (2). Therefore, alternative therapies should be considered. Here, we studied the potential efficacy of rifampicin. Rifampicin is one of the most potent broad-spectrum antibiotics. Although clinical trials more than 30 years ago showed its efficacy in treating gonorrhea, rifampicin has never been established in therapy guidelines (3, 4). However, the potential of this treatment is conditioned by the preexistence of rifampicin resistance in the \textit{N. gonorrhoeae} population. In \textit{Mycobacterium tuberculosis}, 95\% of rifampicin resistance is caused by single nucleotide variants (SNVs) in the \textit{rpoB} gene, coding for the \textit{β} subunit of the RNA polymerase (5). Similar impacts of \textit{rpoB} mutations on rifampicin resistance have been highlighted for \textit{Mycobacterium leprae}, \textit{Mycobacterium kansasi}, and other bacterial species such as \textit{Escherichia coli}, \textit{Bacillus subtilis}, \textit{Staphylococcus aureus}, and \textit{Neisseria meningitidis} (6–8). Therefore, we decided to use these SNVs as a proxy to investigate the frequency of rifampicin resistance in Austrian \textit{N. gonorrhoeae} clinical strains. In a context where multidrug resistance of \textit{N. gonorrhoeae} poses a major threat of treatment failure, such data would help to evaluate the benefit of considering rifampicin as an alternative gonorrhea treatment.
RESULTS

*Neisseria gonorrhoeae* isolates from symptomatic and asymptomatic patients were either provided by 16 Austrian laboratories or cultured at the National Reference Centre (NRC) for Gonococci from clinical specimens (see Table S1 in the supplemental material). Phenotypic AMR was assessed with Etest (bioMérieux, Marcy l’Etoile, France, and Liofilchem, Roseto degli Abruzzi, Italy) according to EUCAST guidelines (9). Approximately 50% of the samples were collected in the province of Vienna, but the collection included isolates from all Austrian provinces (Fig. 1A).

Since 2016, whole-genome sequencing (WGS) has been performed on all isolates collected at the NRC. DNA extraction, library preparation, and Illumina sequencing have been described elsewhere (10). A total of 1,568 *N. gonorrhoeae* isolates collected between 2016 and 2020, for which sequencing data were available, were included in this study. Data on phenotypic AMR testing were available for 1,268 of them.

**FIG 1** *rpoB* single nucleotide variants (SNV) found in the Austrian *N. gonorrhoeae* isolates. (A) Number of isolates according to the year of isolation and the province of origin (*n* = 1,268). (B) Number of isolates carrying *rpoB* SNVs associated with high levels of rifampicin resistance (high res SNV), other nonsynonymous SNVs, and synonymous SNVs, according to the year of isolation (*n* = 1,568). (C) Evolution of the number of resistant isolates over time. Solid lines indicate the 13-week moving average of the number of isolates classified as resistant (*n* = 1,268). Trends over time (obtained by linear regression) are represented by the dashed lines.

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A reference rpoB sequence was obtained from the complete genome of N. gonorrhoeae strain 35/02 (accession no. NZ_CP012028.1). Isolates’ rpoB sequences were extracted from WGS data using SeqSphere+ (Ridom, Muenster, Germany), with a minimum of 99% alignment and 90% identity to the reference sequence. Extracted sequences were searched for SNVs with the SeqSphere+ tool, and 223 SNVs were found, including 33 nonsynonymous SNVs (Table 1). Thirty of 33 SNVs were found in only one or two isolates. Thirty-two isolates, one for each SNV combination that included at least one nonsynonymous SNV, were unfrozen and cultured. Rifampicin Etests (bioMérieux) were performed for the 26/32 viable isolates, and results are presented in Table 1. No results were obtained for 6 SNVs, and 20 SNVs were found only in isolates susceptible to rifampicin. Two SNVs were associated with a high level of rifampicin resistance (minimum inhibitory concentration [MIC] of >32 mg/L): C1657A/His553Asn and T1679C/Leu560Ser. These two SNVs correspond to His526Asn and Leu533Ser in E. coli and were described to confer rifampicin resistance in several bacterial species (11, 12). To assess the impact of genetic backgrounds on rifampicin resistance associated with C1657A, 32 isolates corresponding to 31 different sequence types were tested for rifampicin resistance. All of them had a MIC of >32 mg/L, confirming the association between C1657A and high levels of rifampicin resistance in N. gonorrrhoeae.

| Nucleotide | Reference Base | Reference No. | Reference % | Variant Base | Variant No. | Variant % | MIC (µg/mL) | No. of isolates tested |
|------------|----------------|--------------|-------------|--------------|-------------|-----------|--------------|------------------------|
| 5          | A              | 1,567        | 99.9        | G            | 1           | 0.1       | 1            | >32                    |
| 226        | T              | 1,566        | 99.9        | C            | 2           | 0.1       | 1            | >32                    |
| 239        | G              | 1,558        | 99.4        | A             | 10          | 0.6       | 1            | >32                    |
| 404        | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 505        | C              | 1,567        | 99.9        | T             | 2           | 0.1       | 1            | >32                    |
| 730        | G              | 1,567        | 99.9        | A             | 1           | 0.1       | 1            | >32                    |
| 743        | G              | 1,567        | 99.9        | A             | 1           | 0.1       | 1            | >32                    |
| 824        | C              | 1,566        | 99.9        | T             | 2           | 0.1       | 1            | >32                    |
| 838        | C              | 1,566        | 99.9        | T             | 2           | 0.1       | 1            | >32                    |
| 881        | A              | 1,566        | 99.9        | C             | 2           | 0.1       | 1            | >32                    |
| 983        | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 1094       | A              | 1,566        | 99.9        | C             | 2           | 0.1       | 1            | >32                    |
| 1323       | C              | 1,566        | 99.9        | A             | 2           | 0.1       | 1            | >32                    |
| 1364       | C              | 1,567        | 99.9        | C             | 1           | 0.1       | 1            | >32                    |
| 1657       | C              | 1,096        | 69.9        | A/T           | 472         | 30.1      | >32          | 32                    |
| 1679       | T              | 1,567        | 99.9        | C             | 1           | 0.1       | 1            | >32                    |
| 1769       | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 2018       | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 2048       | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 2075       | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 2146       | G              | 1,567        | 99.9        | A             | 1           | 0.1       | 1            | >32                    |
| 2309       | C              | 1,567        | 99.9        | T             | 1           | 0.1       | 1            | >32                    |
| 2405       | A              | 1,566        | 99.9        | G             | 2           | 0.1       | 1            | >32                    |
| 3169       | T              | 1,566        | 99.9        | G             | 2           | 0.1       | 1            | >32                    |
| 3523       | G              | 1,567        | 99.9        | A             | 1           | 0.1       | 1            | >32                    |
| 3525       | G              | 1,567        | 99.9        | C             | 1           | 0.1       | 1            | >32                    |
| 3527       | G              | 1,566        | 99.9        | A             | 2           | 0.1       | 1            | >32                    |
| 3563       | A              | 1,567        | 99.9        | G             | 1           | 0.1       | 1            | >32                    |
| 3601       | A              | 1,567        | 99.9        | G             | 1           | 0.1       | 1            | >32                    |
| 3602       | G              | 1,567        | 99.9        | C             | 2           | 0.1       | 1            | >32                    |
| 3732       | A              | 1,566        | 99.9        | C/G           | 2           | 0.1       | 1            | >32                    |
| 3800       | C              | 1,566        | 99.9        | T             | 2           | 0.1       | 1            | >32                    |
| 3937       | G              | 1,565        | 99.8        | A             | 3           | 0.2       | 1            | >32                    |

For each variant, this table gives the number and frequency (%) of isolates carrying the reference and variant nucleotide/amino acid. Results of rifampicin Etests and the number of tested isolates are indicated in the last columns. SNVs associated with high or low levels of rifampicin resistance are indicated in boldface and italic, respectively.

aa, amino acid; NA, not available.

One isolate containing SNVs 226, 881, 2018, 3169, 3527, 3601, and 3602 with a MIC of 0.19 µg/mL.

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One isolate containing SNVs 505 and 1094 with a MIC of 0.19 µg/mL.
gonorrhoeae. C1657A and T1679C were found in 473 isolates, which corresponded to 30% of the isolates (Fig. 1B). This proportion was stable over time (Fig. 1C). Five other nonsynonymous SNVs were associated with rifampicin MICs between 0.25 mg/L and 0.5 mg/L. However, given the absence of standardized thresholds for rifampicin resistance in N. gonorrhoeae, the possible involvements of other resistance genes, and the unknown clinical relevance, these SNVs were not analyzed any further in this study.

Phylogenomic relatedness between the isolates was assessed using a local core genome multilocus sequence typing (MLST) scheme previously described (13). The distribution of the isolates was compared with the combination of SNVs carried by their rpoB gene and their potential impact on rifampicin resistance (Fig. 2). Isolates with similar SNV combinations grouped in close branches. Particularly, isolates carrying C1657A or T1679C (indicated as “high-AMR SNVs”) were found in three main branches. This correlation between rpoB variants and population structure suggested a limited number of introductions of rifampicin resistance in Austrian N. gonorrhoeae strains and a mainly vertical evolution of the rpoB gene.

The association between rifampicin resistance and resistance to other antimicrobials was tested using univariate analysis. Measures of association (odds ratio [OR]) and significance of the association (P value < 0.05) are shown in Table 2. Isolates carrying rpoB mutations associated with rifampicin resistance were more likely to be resistant to azithromycin (OR = 22 [95% confidence interval, 10–48.1]), ciprofloxacin (OR = 15 [10.7–20.9]), penicillin (OR = 4.28 [2.74–6.7]), and tetracycline (OR = 7.62 [5.55–10.5]) (Table 2 and Fig. 3).
The DISCUSSION section of the document begins by summarizing the findings of the study, noting that despite the widespread resistance to azithromycin, the authors found that rifampicin resistance was low. They also discuss the implications of their findings for the use of rifampicin as an alternative treatment for gonorrhea.

In terms of MATERIALS AND METHODS, the authors describe the collection and storage of N. gonorrhoeae isolates, as well as the methods used for genomic analysis. They detail the use of rpoB SNVs as a proxy for rifampicin resistance and the identification of nonsynonymous SNVs as a proxy for ciprofloxacin resistance. The study also includes the identification of SNVs as a proxy for tetracycline resistance.

The table provided in the document outlines the association of AMR with rifampicin resistance in N. gonorrhoeae isolates (n = 1,268). The table shows the number of resistant and susceptible isolates for each antimicrobial category, along with the odds ratio (OR) and 95% confidence interval (CI) for univariate analysis.

The authors conclude that rifampicin resistance is not a major concern in the Austrian N. gonorrhoeae population, and that rifampicin should not be considered as an alternative treatment for gonorrhea in Austria, neither alone nor in combination with other AMR.
and applying default software parameters. A 1,524-locus cgMLST scheme and a 463-locus accessory target scheme were obtained, as described in a previous publication (13). Using the distance matrix of the cgMLST analysis, a neighbor-joining tree was computed using SeqSphere+ (Sattath and Tversky’s method). The computed tree was exported in Newick Tree Format and loaded into R to compute dendrograms (packages ggplot [19], ggpubr [20], ape [21], and ggtree [22]).

Data availability. Raw reads were deposited in the Sequence Read Archive (SRA) under project no. PRJNA771206.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
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