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Using a public database of *Neisseria gonorrhoeae* genomes to detect mutations associated with zoliflodacin resistance

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Running Title: Zoliflodacin resistance in *N. gonorrhoeae*

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Synopsis

Background: Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* is an urgent global health threat. Zoliflodacin is a novel antibiotic undergoing clinical trials for the treatment of gonorrhea. While there are limited data regarding zoliflodacin resistance in *N. gonorrhoeae*, three amino acid mutations have been associated with increased MICs to zoliflodacin.

Objective: To determine the prevalence of three amino-acid mutations associated with zoliflodacin resistance within a large, public database of nearly 13,000 *N. gonorrhoeae* genomes.

Methods: PathogenWatch is an online genomic epidemiology platform with a public database of *N. gonorrhoeae* genomes. That database was used to extract *gyrB* sequence data and a basic local alignment search tool (BLAST) search was performed to identify any of the three amino-acid mutations in GyrB that are associated with increased zoliflodacin MICs: D429N, K450N, or K450T. As a control for the search methodology, all GyrA sequences were also extracted and S91F mutations were identified and compared to the PathogenWatch database.

Results: In total, 12,493 *N. gonorrhoeae* genomes from the PathogenWatch database were included. Among those genomes, none were identified that harbored any of the three mutations associated with increased zoliflodacin MICs. One genome was identified to have a mutation at position 429 in GyrB (D429V).

Conclusions: The findings suggest the prevalence of those three mutations associated zoliflodacin resistance in *N. gonorrhoeae* are very low. However, further research into the mechanisms of zoliflodacin resistance in *N. gonorrhoeae* is needed. Genomic epidemiology platforms like PathogenWatch can be used to enhance the global surveillance of AMR.

Background

Neisseria gonorrhoeae is the second most common bacterial sexually transmitted infection (STI), causing approximately 87 million new infections per year worldwide.[1] Antimicrobial resistance (AMR) in *N. gonorrhoeae* is an urgent global health threat, as the pathogen has developed resistance to every class of antibiotics used for its treatment.[2] Ceftriaxone is the final remaining empiric option for gonococcal treatment globally, but strains with resistance to ceftriaxone have recently emerged and treatment failures have occurred.[3-5] New antimicrobial therapies are urgently needed to combat AMR in *N. gonorrhoeae*.

Zoliflodacin is a novel antibiotic of the spiropyrimidinetrione class that targets the B subunit of DNA gyrase (GyrB).[6, 7] Zoliflodacin is bactericidal and has exhibited potent *in vitro* antimicrobial activity against wildtype, multidrug resistant, and extensively drug resistant strains of *N.* gonorrhoeae, with MICs ranging from ≤0.002 to 0.25 mg/L.[8] Among 199 consecutive clinical isolates from Thailand and South Africa, which included 177 ciprofloxacin-resistant isolates, the zoliflodacin MICs ranged from 0.004 to 0.25 mg/L, with a modal MIC of 0.064 mg/L.[9] Similar findings were seen among 873 clinical isolates from 21 European countries, where the zoliflodacin MICs ranged from ≤0.002 to 0.25 mg/L and the modal MIC was 0.125.[10] Following promising results of a phase II trial, a phase III clinical trial of zoliflodacin for treatment of uncomplicated *N. gonorrhoeae* is currently

underway (ClinicalTrials.gov: NCT03959527).[11] As the clinical use of zoliflodacin approaches, a better understanding of the mechanisms for the development of resistance in *N. gonorrhoeae* is critical. Thus far, three amino acid mutations have been associated with higher zoliflodacin MICs. Those mutations were identified through *in vitro* selection of resistance and are located within GyrB: D429N, K450N, or K450T.[12, 13] The presence of one of those mutations was associated with a 4-fold to 16-fold increase in zoliflodacin MICs.[12, 13] While there are no established clinical breakpoints for zoliflodacin, ≥0.5 mg/L was used to define resistance in the Phase 2 clinical trial, although no isolates were found to be resistant.[8, 11]

PathogenWatch (https://pathogen.watch/) is a public database of bacterial whole genome sequences that was created to facilitate genomic epidemiology. The platform includes genomic data from a curated global collection of nearly 13,000 *N. gonorrhoeae* isolates with metadata and includes tools that enable detection of AMR determinants and prediction of AMR based on genomic data.[14] A number of genes and point mutations associated with AMR in *N. gonorrhoeae* can be detected and reported using the PathogenWatch platform. However, that platform does not yet include the GyrB mutations associated with zoliflodacin resistance.

Our objective was to use the PathogenWatch database to determine the prevalence of mutations known to be associated with resistance to zoliflodacin within the global collection of *N. gonorrhoeae* isolates.

Materials and methods

Whole Genome Sequence Data. All whole genome sequence data from 12,943 *N. gonorrhoeae* isolates, publicly available on November 17, 2020 on the PathogenWatch database were included.[15]

Mutation Analysis. The DNA sequences of the *gyrB* genes were extracted from the EzBioCloud database (https://www.ezbiocloud.net/) using the *N. gonorrhoeae* FA 1090 strain as the reference. To perform a control experiment for the search strategy, the *gyrA* gene was also extracted from the downloaded *N. gonorrhoeae* genomes, using the FA 1090 reference strain.

A Basic Local Alignment Search Tool (BLAST v2.2.26+) search was performed to query each of the two genes against the genomes from PathogenWatch with 60% identity/length as a threshold value.[16] The Biopython BLAST IO package was used to parse the result, and subsequent DNA translation to protein was conducted.[17] An in-house python code, which generates counts of different amino acids within a given position value, was used to identify the mutations of interest: S91F in GyrA and D429N, K450N, or K450T in GyrB

(python code and description are available at: https://github.com/smha118/mutation_detecter). One genome contained a partial *gyrA* gene (SRR2736138) and the partial protein sequence was aligned using MUSCLE (v3.8.31).[18] Visual comparison of the alignment confirmed the sequence was partial and did not include the region of interest; thus, we could not verify the mutation type.

Counts of the D429N, K450N, or K450T mutations identified in GyrB were reported. Counts of the S91F mutation identified in GyrA were also reported. The S91F GyrA mutations were compared to what was reported by the PathogenWatch database to serve as controls for the above search strategy.

Results

In total, 12,943 *N. gonorrhoeae* whole genome sequences were downloaded from the PathogenWatch database. The *N. gonorrhoeae* isolates included were collected from 1928 – 2019 and were obtained from 68 countries. Of all the isolates, 68.9% (n = 8,914) were from three countries: United States (n = 3,008), United Kingdom (n = 3,510), and Australia (n = 2,396).

Among all extracted *N. gonorrhoeae* isolates, none contained the D429N, K450N, or K450T amino acid mutations in the GyrB gene.

One isolate was identified to have a mutation at the 429 position of GyrB, specifically a D429V. The isolate was obtained from a male in Japan in the year 2000 and was published as part of a prior analysis (European Nucleotide Archive [ENA] accession number ERR363582).[19] The isolate was susceptible to cefixime with a MIC of 0.094 mg/L, susceptible to azithromycin (MIC = 0.38 mg/L), but had decreased susceptibility to ceftriaxone (MIC = 0.125 mg/L). The isolate was assigned NG-STAR ST-330, with a mosaic PenA allele (10.001) and I312M, V316T, and G545S mutations in PenA. The isolate was resistant to ciprofloxacin (MIC = 1.5 mg/L), with a S91F mutation in GyrA and S87R and S88P mutations in ParC.

Our search strategy identified 41.6% (5395/12,943) isolates with the S91F mutation in GyrA. By comparison, 5392 isolates were labeled as having the S91F GyrA mutation in the PathogenWatch database. The sequences of the three discrepant isolates were manually checked and confirmed to have the S91F mutation in GyrA.

Discussion

Using a public database of whole genome sequences for nearly 13,000 *N. gonorrhoeae* isolates, no mutations known to be associated with resistance to zoliflodacin were identified. The analysis of those whole genome sequences identified one isolate with an amino acid substitution at one of the same locations within the GyrB gene, position 429, that has been associated

with zoliflodacin resistance, but that mutation included a different amino acid substitution than what has previously been reported. Public databases of whole genome sequences for *N. gonorrhoeae* can be used to aid in the genomic surveillance of antimicrobial resistance, including resistance to novel antibiotic therapies.

Data on the evolution of AMR in *N. gonorrhoeae* show that prior to the modern use of antibiotics, *N. gonorrhoeae* did not harbor AMR elements and that resistance has been driven by the widespread use and misuse of antibiotics.[19, 20] Zoliflodacin is a novel antibiotic without widespread use, therefore existing resistance to zoliflodacin is expected to be low. That has been shown in other settings and our findings provide further support for that.[9] However, antibiotic susceptibility testing to zoliflodacin is limited, and data regarding susceptibilities are sparse. In our analysis, phenotypic susceptibility data for zoliflodacin was not available, and thus it was not possible to identify other mutations associated with resistance. Moreover, knowledge about the mechanisms of resistance to zoliflodacin are limited. It is possible that there are additional mutations that confer resistance to zoliflodacin *in vivo* but have not yet been identified.

One isolate from the PathogenWatch database had a mutation at amino acid position 429 in GyrB that has been implicated in zoliflodacin resistance. The susceptibility to zoliflodacin for that isolate was not reported. If that isolate

could be located and tested for antibiotic susceptibility, it would be of high interest to determine if that mutation is associated with an increased MIC to zoliflodacin. Those efforts are currently underway. Interestingly, a search of the UniProt database for the mutations of interest in GyrB of *N. gonorrhoeae* returned one isolate with a D429N.[21] The isolate was included in a report by Stein *et al.* in 1991 and was associated with low-level resistance to nalidixic acid, although with only slightly increased MICs to the other quinolones tested (GenBank Accession Number: M59981).[22] Zoliflodacin susceptibility for that isolate is not known, but would be of high interest.

In our analysis of mutations associated with zoliflodacin resistance, we demonstrated one potential application of the PathogenWatch database. The platform can automatically detect genetic determinants of AMR and make predictions of phenotypic resistance based on those mutations.[14] Currently, the prediction profile does not include mutations associated with zoliflodacin resistance. As zoliflodacin resistance mechanisms are identified, they can be incorporated into existing genomic platforms, like those at PathogenWatch, and be used to enhance AMR surveillance. Additional research into the mechanisms and determinants of zoliflodacin resistance will be important, as its clinical use approaches.

International collaboration is needed to improve the global surveillance of AMR in *N. gonorrhoeae*.[2] Improving open-access to *N. gonorrhoeae* genomic

data will be critical to that mission. Platforms like PathogenWatch can expand the use of genomic epidemiology to improve public health surveillance of AMR in *N. gonorrhoeae* by providing a community-developed, open-access database of isolates and by enabling the use of genomic epidemiology tools without requiring additional expertise in bioinformatics that can be a barrier in many low- and middle-income countries.[14] As the use of whole genome sequencing for *N. gonorrhoeae* is expanding, additional data deposited in public databases like PathogenWatch can be used to improve the platform and to advance our understanding of the transmission and epidemiology of AMR in *N. gonorrhoeae* worldwide.

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