ANTIMICROBIAL RESISTANCE AND WHOLE GENOME SEQUENCING ANALYSIS OF *Neisseria gonorrhoeae* ISOLATES IN BULGARIA, 2019

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Abstract

*Neisseria gonorrhoeae* is evolving into a superbug with resistance to previously and currently recommended antimicrobials for treatment of gonorrhoea, which creates a significant public health concern globally. Antimicrobial resistance (AMR) poses a great threat; it causes longer duration of illness and treatment, and increases health care costs and the social, psychological and financial burden to families and societies. In response to this worrying situation laboratory research should be strengthened with implementation of newer molecular methods for monitoring and detecting AMR, and evaluation of the correlation of obtained genetic data with corresponding phenotypes.

This study aimed to implement whole genome sequencing analysis for the first time of *N. gonorrhoeae* isolates from Bulgaria and to acquire molecular epidemiological and AMR information linked with phenotypic resistance data.

Although the tested strains were susceptible to current recommended dual therapy, genetic analysis showed the presence of several mutations responsible for reducing susceptibility to third generation cephalosporins, namely the...
presence of genotype G1407 strains in the Bulgarian population. Regarding previously recommended antimicrobials, penicillin resistance and intermediate sensitivity to tetracyclines was demonstrated in all strains, and fluoroquinolone resistance in two strains.

The obtained results showed that the use of whole genome sequencing was feasible, could describe current circulating gonococcal strains and predict and infer transmission of antimicrobial resistance. Therefore, gonococcal infections prevention and control programmes will be aided to target interventions where needed and to revise treatment guidelines that will help improve patient care. **Key words:** Neisseria gonorrhoeae, whole genome sequencing, antimicrobial resistance

**Introduction.** The World Health Organization (WHO) estimated in 2016 that there were 86 million new gonococcal infections among people 15–49 years old worldwide [1]. *Neisseria gonorrhoeae* has developed resistance to almost all of the antimicrobials previously used for the treatment of gonorrhoea, including penicillins, tetracyclines, and fluoroquinolones [2]; the remaining treatment options are now threatened by the emergence of resistance to third-generation cephalosporins and azithromycin [3,4]. In response to that global threat both WHO and the European Centre for Disease Prevention and Control have called for research into the development not only of alternative gonococcal treatment options but also of enhanced *N. gonorrhoeae* surveillance systems [5,6]. There is therefore an urgent need to strengthen *N. gonorrhoeae* surveillance in Bulgaria in order to guide current gonorrhoea treatment regimens and to maintain effective infection control of the disease. Whole genome sequencing (WGS) represents useful tool to adequately assess the emergence, spread and persistence of resistant strains and genetic determinants of antimicrobial resistance locally, nationally and internationally over time and provide additional information, e.g., antimicrobial resistance (AMR) prediction [7,8].

The aim of this study was to implement WGS, in conjunction with linked phenotypic antimicrobial susceptibility data, and thus to enhance national gonococcal antimicrobial surveillance by identifying emerging AMR, monitoring AMR trends, and advising improvements of empirical treatment guidelines.

**Materials and methods.** *N. gonorrhoeae isolates.* Three *N. gonorrhoeae* isolates were cultured from patients with gonococcal infection of the lower genitourinary tract in 2019 and were deposited at the National Center of Infectious and Parasitic Diseases with the identification codes 0025-19, 2938-19, and 3393-19.

Antimicrobial susceptibility testing was done by determination of the minimum inhibitory concentrations (MICs) with Etest (bioMérieux) for cefixime, ceftriaxone, azithromycin, ciprofloxacin, tetracycline, and benzylpenicillin. Clinical breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied for interpretation of MICs and an ECOFF 1 mg/L
was used for testing purposes with aim to detect acquired resistance mechanisms for azithromycin as advised by EUCAST. MICs of 0.047–0.94 µg/ml was used as the cut-off for decreased susceptibility to ceftriaxone and cefixime.

**Whole-genome sequencing of the *N. gonorrhoeae* isolates.** DNA extracts from *N. gonorrhoeae* isolates were obtained with the PureLink™ Genomic DNA mini kit (Invitrogen, USA), according to manufacturer’s instructions. DNA extracts were then multiplexed and sequenced on the Illumina MiSeq platform at the National Center of Infectious and Parasitic Diseases. The obtained sequence reads were processed with whole-genome sequencing pipeline Gen2Epi that assembles short reads into full scaffolds and assigns molecular epidemiological and AMR information to the assembled genomes as previously described [9]. Thereby we derived multilocus sequence typing (MLST) sequence types, NG-MAST sequence types, the presence of plasmids and known antimicrobial resistance determinants. For detection of resistance determinants were used DNA sequences of 8 genes (*penA, mtrR, porB, ponA, gyrA, parC, rpsJ* and 23S rRNA) associated with resistance to beta-lactam antimicrobials, macrolides, tetracycline or fluoroquinolones [2].

**Results. Antimicrobial susceptibility testing.** An analysis of the susceptibility to antimicrobials currently recommended as first line treatment for gonococcal infection showed susceptibility to azithromycin, ceftriaxone and cefixime. However, strain 3393-19 showed decreased susceptibility to cefixime. In addition, all isolates were resistant to benzylpenicillin and intermediate to tetracyclines. Furthermore, strains 0025-19 and 3393-19 displayed resistance to ciprofloxacin (Table 1).

![Table 1](image)

Parameters of susceptibility of the analyzed *N. gonorrhoeae* strains to antimicrobials and their interpretation according to EUCAST

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>0025-19</th>
<th>2938-19</th>
<th>3393-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>0.047 (no resistance)</td>
<td>0.19 (no resistance)</td>
<td>0.5 (no resistance)</td>
</tr>
<tr>
<td>Cefixime</td>
<td>0.016 (S)</td>
<td>&lt; 0.016 (S)</td>
<td>0.094 (DS*)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&lt; 0.016 (S)</td>
<td>&lt; 0.016 (S)</td>
<td>0.023 (S)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1 (R)</td>
<td>&lt; 0.016 (S)</td>
<td>8 (R)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1 (I)</td>
<td>1 (I)</td>
<td>1 (I)</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>2(R)</td>
<td>48(R)</td>
<td>4(R)</td>
</tr>
</tbody>
</table>

S – Susceptible, standard dosing regimen, I – Susceptible, increased exposure, R – Resistant

*Decreased Susceptibility – MICs of 0.047–0.94 mg/L was used as the cut-off for decreased susceptibility to ceftriaxone and cefixime*
Molecular epidemiological characteristics. MLST and NG-MAST sequence types and genogroups are shown in Table 2. Notably strain 3393-19 belonged to the multidrug-resistant NG-MAST genogroup G1407.

<table>
<thead>
<tr>
<th>Sequence types schemes</th>
<th>Sequence types of N. gonorrhoeae strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0025-19</td>
</tr>
<tr>
<td>NG-MAST sequence types</td>
<td>11047</td>
</tr>
<tr>
<td>NG-MAS genogroups</td>
<td>G225</td>
</tr>
<tr>
<td>MLST sequence types</td>
<td>7827</td>
</tr>
</tbody>
</table>

Genetic determinants of antimicrobial resistance in N. gonorrhoeae. Plasmid-mediated penicillin and tetracycline resistance. Cryptic plasmids were identified for each strain. Additionally, Africa type-Neisseria gonorrhoeae plasmid, carrying the blaTEM gene was found for strain 2938-19. No conjugative plasmids were detected (Table 3).

<table>
<thead>
<tr>
<th>Genes (proteins)</th>
<th>Resistance to antimicrobials</th>
<th>Genes and nucleotide polymorphisms (amino acid substitutions)</th>
<th>0025-19</th>
<th>2938-19</th>
<th>3393-19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasmid-mediated resistance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>blaTEM (β-lactamase)</td>
<td>β-lactams</td>
<td>–</td>
<td>blaTEM</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>tetM gene</td>
<td>tetracyclines</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromosomally mediated resistance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>penA (PBP1)</td>
<td>β-lactams</td>
<td>penAType XIII NonMosaic A501V, A517G</td>
<td>L421P</td>
<td>WT</td>
<td>L421P</td>
</tr>
<tr>
<td>penA (PBP2)</td>
<td>β-lactams</td>
<td>penAType II NonMosaic A517G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S rRNA</td>
<td>macrolides</td>
<td></td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>gyrA</td>
<td>fluoroquinolones</td>
<td></td>
<td>S91F</td>
<td>S91F</td>
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</tr>
<tr>
<td>parC</td>
<td>fluoroquinolones</td>
<td></td>
<td>D86N</td>
<td>WT</td>
<td>S87R</td>
</tr>
<tr>
<td>rpsJ (S10)</td>
<td>tetracyclines</td>
<td></td>
<td>V57M</td>
<td>V57M</td>
<td>V57M</td>
</tr>
<tr>
<td>porB (PorB1b porin)</td>
<td>β-lactams tetracyclines</td>
<td></td>
<td>G120K</td>
<td>A121D</td>
<td>G120K</td>
</tr>
<tr>
<td>mirR (MtrCDE efflux pump)</td>
<td>β-lactams tetracyclines</td>
<td>– 35A Del G45D</td>
<td>A39T</td>
<td>– 35A Del</td>
<td></td>
</tr>
</tbody>
</table>

Decreased influx and increased efflux of antimicrobials

Legend: “–” – the gene was not found; “WT” – wild-type sequence

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Chromosomally mediated penicillin and cephalosporin resistance. The sequences of chromosomal genes encoding penicillin binding proteins (PBPs) were found to carry several nucleotide substitutions that significantly reduce susceptibility to β-lactams. Thus, strains 0025-19 and 3393-19 carried the mutation L421P in ponA gene encoding PBP₁, resulting in reduced affinity to penicillins compared to the wild-type protein. Even greater changes were detected in strain’s 3393-19 penA gene sequences (encoding PBP₂), reflecting the concept of mosaic-like structure formed due to a genetic recombination with synanthropic commensals. More amino acid substitutions, namely A501V, A517G and N512Y, were found in the PBP₂.

Macrolide resistance. The results of the search for A2059G and C2611T mutations in 23S rRNA, which disrupt the interaction between macrolide antibiotics and their target (the peptidyl transferase centre) in domain V of 23rRNA, indicate that all three strains were wild type.

Fluoroquinolone resistance. In strains 0025-19 and 3393-19, gyrA gene was found to contain S91F and D95G amino acid substitutions associated with fluoroquinolone resistance. The parC gene in these strains possessed D86N and S87R substitutions, respectively. Wild type gyrA and parC genes were present in strain 2938-19.

Chromosomally mediated tetracycline resistance. A point mutation causing the amino acid substitution V57M in ribosomal protein S10 of the 30S ribosomal subunit was found in the chromosomal rpsJ gene in all three analyzed genomes.

Decreased influx and increased efflux. The penB gene encoding the outer membrane porin PorB1b is also involved in the emergence of resistance to β-lactams in N. gonorrhoeae. The amino acid substitutions G120K and A121D/A121N in this protein reducing membrane permeability for hydrophilic antibiotics were detected in strains 0025-19 and 3393-19, whereas 2938-19 strain carried no substitutions.

Mutations within the promoter and/or coding region of the mtrR repressor may end in the overexpression of the MtrCDE efflux pump. The deletion – 35A Del of the mtrR promoter region was found in strain 0025-19 and 3393-19. Furthermore, a G45D or A39T mutation in the mtrR coding region was detected in strain 0025-19 and 2938-19.

Discussion. We report the findings of the first study to supplement the Bulgarian gonococcal antimicrobial surveillance with WGS analysis to provide enhanced surveillance of gonorrhoea. This approach has not only provided information on molecular epidemiology of circulating strains, but also provides insight into the distribution of genetic determinants of antimicrobial resistance within the country.

We found different for each strain NG-MAST and MLST sequence types regarding molecular epidemiology, which should well predict different phenotypic patterns of susceptibility. The results suggest that circulating strains represent
mixed population from two distinct lineages – multi-resistant (strain 0025-19 and 3393-19) and multi-susceptible lineage (strain 2938-19) \[10\]. Notably strain 3393-19 belonged to MLST ST1901 and NG-MAST G1407 genogroup.

MLST ST1901 and NG-MAST G1407 genogroup are known to be distributed globally and are documented in studies as responsible for most of the decreased susceptibility and resistance to cephalosporins in Europe, Asia, North America, etc. \[11-15\]. ST1407 and related sequence types have also caused most of the treatment failures with third generation cephalosporins in EU/EEA countries \[3,16-18\]. Therefore, it is not surprising to find such sequence types in Bulgaria, but their circulation should be closely monitored in order to prevent epidemic outbreaks and spread of resistant gonococcal infections, and to limit the threat of possibly incurable gonorrhoea.

We assessed the association between antimicrobial resistance genotype and phenotype. The data presented here offer good correlation between the detected genetic determinants of resistance and the observed MICs to the respective antimicrobials. All three strains were susceptible to the currently recommended \[19\] dual treatment (i.e. ceftriaxone plus azithromycin) as predicted by molecular analysis. None of the specific mutations (A2059G, C2611T) of the macrolide target, 23S rRNA, were discovered in any of the examined strains. Nevertheless regarding cephalosporin resistance in strain 3393-19 we have found mosaic \textit{penA} XXXIV allele and additional mutations in the promoter region of the \textit{mtrR} repressor (-35A Del) and in major outer membrane porin PorB1b (G120K, A121N) which was in concordance with the increased MIC found for cefixime. Noteworthy for strain 0025-19 despite the mutations found in the non-mosaic \textit{penA}, \textit{mtrR} and \textit{porB} genes, no elevated MICs for cefixime and ceftriaxone were detected. That denotes those mosaic \textit{penA} genes together with \textit{mtrR} and \textit{porB} genes alterations, are important for decreased susceptibility/resistance to third generation cephalosporins but also hint at the complex interactions and interplay with resistance determinants that increase the efflux and decrease the influx of cephalosporins.

On antibiotics that were previously recommended as a first-line treatment we found all three strains resistant to benzylpenicillin. Strain 2938-19 has plasmid-mediated high-level resistance, whereas strain 0025-19 and 3393-19 harboured a single missense mutation in the \textit{ponA} gene (L421P) that encodes PBP1. Regarding tetracyclines no plasmid-mediated resistance was found. However, all three strains had mutated \textit{rpsJ} gene (V57M) which agreed with slightly increased MICs, reported as “I – Susceptible, increased exposure” as by EUCAST definition. In the antimicrobial susceptibility testing to fluoroquinolones, strains 0025-19 and 3396-19 were found to be resistant to ciprofloxacin and accordingly carried missense mutations at codon 91 and 95 in \textit{gyrA} gene and D86N or S87R mutations in \textit{parC} gene in addition.

In summary, associations of the analyzed genes to penicillin, cephalosporin, tetracycline, fluoroquinolone, and macrolide resistances have been already well
characterized [2] and the performed genome analysis addresses reliably gonococcal antimicrobial resistance and can be used to identify early enough emerging outbreaks and to monitor the national dissemination of resistant \textit{N. gonorrhoeae} isolates.

WGS has many benefits over conventional molecular epidemiological typing methods, which are suboptimal for molecular epidemiology. For example, WGS has the superior resolution versus NG-MAST and MLST, and linked to epidemiological data, has the potential to allow early identification of novel outbreaks or high-risk clones (e.g., as defined by antimicrobial resistance). In addition, the WGS analysis permits the detection of the genetic determinants of resistance to multiple classes of currently or previously recommended as first-line treatment antimicrobials. This highlights the prospect of WGS analysis as a tool to predict antimicrobial resistance both for the needs of personalized medicine and for use in public health interventions and programmes. The increase of decreased susceptibility and resistance to third generation cephalosporins, including the emergence of ceftriaxone-resistant gonorrhoea, warrants consideration of novel approaches such as WGS analysis to prevent or prepare for the possibility of untreatable gonorrhoea. For molecular epidemiology and gonococcal antimicrobial resistance surveillance at national level, WGS should soon be the method of choice. WGS becomes more cost-effective every year, and platforms such as whole-genome sequencing pipeline Gen2Epi provide automated analysis of WGS data tailored specifically for \textit{N. gonorrhoeae}.

**Conclusions.** The findings of this study could contribute to our understanding on spread of \textit{N. gonorrhoeae} and its resistant strains at national level. The use of whole genome sequencing analysis for surveillance, outbreak analysis, personalized medicine and development of diagnostic tests could provide a crucial aid to the design and implementation of evidence-based strategies for gonococcal infection control and prevention.

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Two cases of verified clinical failures using internationally recommended first-line

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