Ph.D. Dissertation Research Protocol

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Title: Molecular epidemiology of *Neisseria gonorrhoeae* in Greece. Genotypic characterization of resistant strains and analysis of resistance mechanisms

INTRODUCTION

Neisseria gonorrhoeae is the pathogenic cause of gonorrhea, one of the major sexually transmitted diseases of bacterial etiology. According to the most recent estimation of the World Health Organization, the global impact of gonorrhea reached, in 2012, 78 million cases, of which 4,7 million were detected in Europe and another 4,5 million in the region of East Mediterranean (5). Because of the impact of the disease and its serious implications if not timely treated, the control of gonorrhea constitutes a matter of major importance for Public Health.

The gonococcus is inherently sensitive to antibiotics, but at the same time particularly capable in developing new properties, owing to its genetic advantages (high rate of mutations, natural ability to incorporate absorb DNA by genetic transformation), which confer a strong adaptive capacity to the changing environmental conditions.

In summary, the main resistance mechanisms that have been identified in gonococcus thus far (10, 12, 13) are:

(**a**) The acquisition of plasmids that lead to high-level resistance either to penicillin, provided that they carry genes responsible for the production of penicillinase, or to tetracycline , when they carry the *tetM* gene, which is coding for a protein that is binding to the ribosome and protects it from the effect of tetracycline. (**b**) The acquisition of conjugative transposons that are responsible for the high-level resistance to macrolides, carrying *erm* genes or *mef* genes. The *erm* (erythromycin methylase genes) is coding for a methylase that protects the 23S subunit of the ribosome from the effect of macrolides, while the *mef* (macrolide efflux genes) are responsible for the activation of a specific efflux pump that removes macrolides from the gonococcus cell. (**c**) Point mutations that affect the ribosomal proteins (16S, 5S) and lead to high-level resistance to spectinomycin, impeding its binding connection to the ribosome, as well as other ribosomal mutations that lead to high-level resistance to macrolides, such as mutations within the area V of the 23SrRNA gene or to genes *rplD* and *rplV* that are coding for the ribosomal proteins 4 and L22, respectively. (**d**) Accumulation of mutations to the *gyrA* and *parC* genes, which cumulatively lead to high-level resistance to fluoroquinolones, causing an alteration to the structure of gyrase A and topoisomerase IV enzymes, that are the target of quinolones in the gonococcus cell. (**e**) Individual mutations or mosaicism of the *penA* gene, that is located on the chromosome and codes for penicillin-binding protein 2 (PBP-2) which constitutes the target of β-lactam antibiotics in the gonococcal cell. The individual mutations as well as the mosaicism of the *penA* gene, lead to the production of mutated PBP-2 exhibiting altered binding properties toβ-lactam antibiotics. (**f**) Mutations on chromosomal genes, such as *por* genes (*penB* etc.), that are related to permeability impairment as well as the *mtr* genes

(RCDE) of the *mtr* operon that are involved in the regulation of the non-specific efflux pump of gonococcus. These mutations are responsible for the alteration in the expression level of the efflux pump, changes in the intracellular concentration of antibiotics and consequently inhibition of access of the antibiotic to the targets within the gonococcus cell. The latter leads to low level non-specific resistance to a variety of antibiotics (although sometimes reaches clinically significant levels), such as penicillin, tetracycline, macrolides, chloramphenicol etc. (10, 12, 13).

The emergence and subsequent wide spread of most of the above resistance mechanisms have excluded the antibiotics, such as penicillin, tetracycline, erythromycin, chloramphenicol from the empirical therapy, while in most countries, including Greece, the newest fluorinated quinolones cannot be prescribed due to high levels of resistance. The emergence –in 1998 in Japan– of *N. gonorrhoeae* strains with reduced susceptibility to 3rd generation cephalosporins (3CG) and, most importantly their global spread, has made the so-called "untreatable" gonorrhea a risk which is now considered as one of the major public health problems (1, 7, 10, 11,13). Surveillance of the epidemiological and microbiological traits of gonorrhea and monitoring of gonococcal resistance to antibiotics, is imperative in order to control the dissemination of *N. gonorrhoeae* resistant strains.

The use of high-throughput sequencing technologies (next-generation sequencing, NGS) allows the identification of the known resistance genes/mechanisms, but at the same time facilitates the identification of new mutations/resistance genes. Apart from the improved ability to identify the resistance profile, the genomic analyses provide tools that can be used for the a) typing of *N. gonorrhoeae* strains, b) determination of the phylogenetic relationships among the *N. gonorrhoeae* population, c) the identification of new resistant lineages in the *N. gonorrhoeae* population and d) assessment of the mode of dissemination of *N. gonorrhoeae* strains.

THE OBJECTIVE of the present doctoral thesis, which will be conducted in the Molecular Microbiology and Immunology laboratory, of the Department of Biomedical sciences of the University of West Attica in collaboration with the National Reference Center for *Neisseria gonorrhoeae* (NRCNG) of the Hellenic Pasteur Institute, is to contribute to the molecular epidemiology and monitoring of resistance trends of *N. gonorrhoeae* in Greece. This will be achieved by epidemiological and microbiological data collection for a three-year period (2020-2022) and comparison to the respective ones from previous years. The monitoring will include the genotypic characterization of all *N. gonorrhoeae* strains, analyses of the underlying resistance mechanisms and genotypic characterization of resistant strains in order to identify the transmission routes and the phylogenetic relationships a well as to determine the gonococcal population structure in Greece.

MATERIAL AND METHODS

Biological material. The *N. gonorrhoeae* isolates originating from an equal number of distinct cases of gonococcal infections from the hospitals and diagnostic centers of Greece (~100-150 strains annually) will be collected in NRCNG during the three-year period of 2020-2022.

Collection – Identification – Culture –Cryopreservation of N. gonorrhoeae strains. For all strains that will comprise the collection of gonococci for the three-year period of 2020-2022 the following tests will be performed: identification in order to confirm the lab diagnosis (Gram-staining) will be based on biochemical system RapID NH System (Remel), serotyping, culture (morphology, oxidase test, growth on selective media) and the detection of the cryptic plasmid of the gonococcus (6). *N. gonorrhoeae* isolates will be cultivated on GCagar with Vitox supplement (Oxoid), with or without antibiotics (VCN supplement, Oxoid) with 18-48h of incubation at 35-37^{o}C, in a humid environment (70%), enriched with CO_2 (5-7%). The strains of the collection will be stored at -80^{o}C, in Brain Heart Infusion Broth with 20% glycerol (glycerol stocks).

Collection and Processing of Epidemiological Data. All strains will be accompanied by the clinical history, based on NRCNG's standard questionnaire (2), which will include demographic, epidemiological and clinical data for the corresponding cases of gonorrhoea infections. The collected data will be statistically analyzed and the correlations among the *N. gonorrhoeae* strains will be examined in order to analyze the transmission routes, the epidemiological parameters that affect their transmission and also to study the social groups that possibly accumulates and preserve resistant strains.

The epidemiological data will be collected anonymized and pseudoanonymized, while only the necessary data will be kept in an encrypted record.

Identification of the Levels of Susceptibility to Antibiotics. The susceptibility profile for eight antibiotics (penicillin G, cefixime, ceftriaxone, tetracycline, azithromycin, spectinomycin, gentamicin, ciprofloxacin) will be determined for all strains. For selected strains with particular resistance/susceptibility phenotypes, the susceptibility testing will be extended to more antibiotics. The levels of resistance will be based on the determination of the minimum inhibitory concentrations (MIC) of antibiotics (Etest, AB-Biodisk, Biomérieux). The clinical breakpoints established by EUCAST (www.eucast.org) will be used for the evaluation of the results and classification of the strains in susceptibility categories.

Characterization of Resistance Mechanisms. The underlying resistance mechanisms will be identified based on their susceptibility phenotype and the use of phenotypic tests as well as genotypic methods that will be applied on a case-by-case approach. The nitrosefin test will be used for the detection of penicillinase-producing (PPNG) strains, the analysis of plasmid content will be applied in the PPNG strains and the strains with high-level resistance to tetracycline (TRNG), the PCR and PCR-RFLP methodology will be used for the detection and characterization of the *tetM* gene in TRNG strains, PCR and analysis of nucleotide sequence will be performed for the characterization of gyrase and topoisomerase IV mutations in QRNG strains, the characterization of the resistance mechanism to macrolides (high-level resistance to azithromycin) as well as the characterization of the *penA* gene for strains resistant to 3CG (CDS).

Strain typing. All strains will be classified to serotypes by using the GC monoclonal antibody system against the gonococcus's outer membrane main protein I (Phadebact GC Serovar Panel, MKL Diagnostics AB, Stockholm, Sweden) (8,9). Groups of isolates with clonal indications and/or strains that require further study will be analytically characterized by the application of molecular typing methods. More specifically, for the comparison of possibly clonally linked isolates, the method of Pulsed Field Gel Electroforesis (PFGE) will be used (3,

9), while for the characterization of new strains or representative strains the methodologies of NG-MAST (*N. gonorrhoeae Multi Antigen Sequencing Typing*) and MLST (*Multi Locus Sequencing Typing*) will also be used. The Ng-MAST analyzes the sequences of hypervariable genes and is used for the evaluation of genetic similarity of strains that are isolated within a short period of time, aiming at the detection of epidemic clones, whereas the MLST identifies the sequences of housekeeping conserved genes of *N. gonorrhoeae* , aiming at the evaluation of phylogenetic relations among the strains (4).

Analysis of genomes by Next Generation Sequencing (NGS). NGS methodology will be used in order to determine the genomes of *N. gonorrhoeae* strains within clones that are predominant in the community, the resistance genes (resistome) and pathogenicity genes will be identified. The total genomic DNA of *N. gonorrhoeae* strains will be isolated and its quality and quantity will be checked. Genomic libraries of an average size of 400 bp will be created and next generation sequencing in an Ion Torrent S5 platform will be performed. The bioinformatic analyses, through the use of software packages, will follow. The phylogenetic relationships between the isolates will be studied through the analysis of the genomes, revealing the population structure of gonococci in Greece.

PROSPECTIVE RESULTS

The prospective results of the doctoral dissertation include:

- The monitoring of resistance frequencies (resistance of *N. gonorrhoeae* to antibiotics and the types of gonococcus that will be isolated in the three-year term of 2020-2022).
- The evaluation of the microbiological and epidemiological parameters that contribute to the dissemination of resistance.
- The characterization of the underlying resistance mechanisms in *N. gonorrhoeae* strains that are isolated in Greece.
- The characterization of the genomes of the *N. gonorrhoeae* strains that are isolated in Greece.
- The creation of an electronic data base – Correlation of clinical, epidemiological, microbiological and genomic data for *N. gonorrhoeae*.
- Study of the phylogenetic relationships among *N. gonorrhoeae* strains that will be isolated during the three-year term of 2020-2022 and the population structure of gonococci in Greece.

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