Gonorrhoea infection: Antimicrobial susceptibility and molecular typing of *Neisseria gonorrhoeae* isolates

PhD thesis

Brunner Alexandra, MD

Semmelweis University
Doctoral School of Clinical Medicine

Supervisor:	Eszter Ostorházi, MD, PhD
Official reviewers:	István Sziller, MD, PhD Artúr Beke, MD, PhD
Head of the final examination comittee:	Károly Cseh, MD, DSc
Members of the final examination committee:	Zsuzsanna Szalai, MD, PhD Zsolt Melczer, MD, PhD

Budapest
2017

Introduction

Gonorrhoea, caused by *Neisseria gonorrhoeae*, is the second most commonly reported bacterial STI according to European surveys. It remains a major public health concern worldwide affecting the quality of life, fertility, transmission of HIV, causing high socioeconomic consequences. According to the increasing frequency of infection, the spread of resistant bacteria, and the lack of recent antibiotic therapy, the problem will increase in the future.

After the millennium, the incidence of infection started to increase. The infection can only be cured with antibiotic therapy, but the number of antibiotic-resistant strains has been increasing in recent decades. Multidrug-resistant and extensively-drug resistant (MDR, XDR) strains that are resistant to the antibiotics of the current therapeutic recommendation, the 3rd generation cephalosporins and azithromycin, have also been published. In 2016, in the United Kingdom, the first therapeutic failure was reported following the use of the first-line treatment, the dual antimicrobial therapy (ceftriaxone and azithromycin). According to the publication of WHO in 2012, it is conceivable that there will be no effective therapies available in the future due to the increasing resistance of strains. Therefore the WHO emphasizes the importance of continuous monitoring of antibiotic susceptibility. This highlights the importance of new therapeutic plans and the introduction of new antibiotics in the treatment of gonorrhea. Knowledge of local susceptibility patterns combined with incidence of various sequence types of *N. gonorrhoeae* is essential to establish the best regional treatment guideline. Among the currently available sequence-based methods *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) is one of the most advantageous, because it is highly discriminatory and reproducible, and the results can be compared objectively between laboratories based on DNA sequence. Phylogenetic studies on the sequence types of a specific region can describe the evolutionary tendencies between STs.

Accordingly, in our studies we have put great emphasis on the measurement of antimicrobial resistance in Hungary over the last few years, with particular reference to the first choice antibiotics, to cephalosporins and azithromycin. NG-MAST was used to investigate the sequence types of strains. According to our knowledge, this was the first survey in Hungary to investigate the relationship between sequence types and associated resistance data in order to create appropriate empirical therapy.

Objectives

A) **1.** My aims were to assess the incidence of gonorrhea infection confirmed by culture and to assess the distribution of infection according to age and gender between 2010 and 2014, at the Department of Dermatology, Venereology and Dermatoocology, at the *Neisseria gonorrhoeae* Reference Laboratory of Semmelweis University.

2. I proposed to investigate the distribution of infection according to anatomical sites, particularly pharynx infection, which is asymptomatic in most cases and plays an important role in the spread of resistant strains due to the transfer of resistant genes between the commensal and pathogenic Neisseria.

3. Since gonococcus became resistant to all drugs recommended for treatment over the last 70-80 decades, it is important to monitor antibiotic sensitivity every year as it is done in the GASP programs worldwide. In 2012 WHO also emphasizes the continuous monitoring of antibiotic resistance. Our study aimed to determine the antimicrobial resistance, especially the resistance to current therapeutic recommendations (3rd generation of cephalosporins and azithromycin).

4. According to the current European guideline, combination therapy with cefriaxone and azithromycin seems to show synergy, and also eradicates *Chlamydia trachomatis* coinfection, which is a relatively common STD under the age of 30. My goal was to assess the rate of chlamydia coinfection at the Department of Dermatology, Venereology and Dermatoocology. Evaluating the results of samples of STD center studied with multiplex RT-PCR, the frequency of C.trachomatis and gonorrhoea infection were analyzed.

B) Use of molecular tests to establish a rational therapy in Hungary.

1. I aimed to characterize 52 N. gonorrhoeae strains from 2013 by NG-MAST typing method to describe the incidence of NG-MAST types in Hungary and compare the results with the European data. Our study is the first representative survey in Hungary that, by typing a sufficient number of strains, is capable of replacing the data gap that has existed for years and may supplement the data of Euro-GASP.

2. In addition, I proposed to analyze 50 azithromycin-resistant strain from 2014 by NG-MAST method and to compare the results with foreign surveys.

3. Another aim was to complete phylogenic analysis of NG-MAST sequence types identified in 2014 to estimate the evolutionary relationships, and to draw conclusions about the successful STs.

Methods

Culturing of *N. gonorrhoeae* bacteria

The isolates were cultured from consecutively symptomatic gonorrhoea patients and from their asymptomatic contacts. For the identification of N. gonorrhoeae, cervical, anal, urethral and pharyngeal swabs were taken and cultured on preheated VCA3 agar (Biomérieux, Budapest, Hungary) and on non-selective PVX chocolate agar (Biomérieux, Budapest, Hungary). Cultures were incubated for 24-72 hours at a temperature of 37°C with 5% carbon dioxide.

Measuring of antimicrobial susceptibility by Epsilometer test

Minimum inhibitory concentrations (MIC; mg/L) of cefixime, ceftriaxone, penicillin, tetracycline, azithromycin, spectinomycin and ciprofloxacin were determined on PVX chocolate agar (Biomérieux, Budapest, Hungary) using MIC strip tests (Liofilchem® s.r.l., Roseto degli Abruzzi, Italy), according to the manufacturer's instructions, and by using a direct colony suspension equivalent to a 0.5 McFarland standard. Testing conditions also included incubation at 36.5°C and 5% carbon dioxide for 24 hours. All results were interpreted by using breakpoints for susceptibility and resistance according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Detection of *C. trachomatis* coinfection by multiplex RT-PCR

DNA isolation was performed directly from the samples taken from the patients by RibospinTM vRD exctraction kit (GeneAll®) according to the manufacturer's instructions. Anyplex™ II STI-7 Detection STD kit (Chemium, Hungary) was used for multiplex RT-PCR according to the manufacturer's (Seegene) instructions.

NG-MAST method

52 strains were selected from *N. gonorrhoeae* strains isolated in 2013 (one index strain from every sexually contact related group). In 2014 50 resistant and intermediately resistant strains were selected for N. gonorrhoeae multiantigen sequence typing (NG-MAST). NG-MAST method is described below:

Culturing Neisseria gonorrhoeae strains

N. gonorrhoeae strains were retrieved from storage at –80 °C on Cryobank breads (Mast Diagnostica, Germany), cultured on VCA chocolate agar, and incubated overnight at 36.5 °C with 5% carbon dioxide.

Isolation of genomic DNA

A turbid suspension of the gonococcal subculture was made in PCR water and DNA was isolated with RibospinTM vRD extraction kit (GeneAll®) in 2013 and DNA-sorb-AM extraction kit (AmpliSens. ®).

Sequencing internal fragments of *porB* and *tbpB*

Polymerase chain reaction (PCR) amplification of the *porB* and *tbpB* gene fragments was performed with *porB* and *tbpB* primers. Primers used in international research were used (Csertex ltd. Budapest). The study was conducted in accordance with international research (Martin IM, 2004) as previously described. The presence of amplified gene fragments was verified by agarose gel electrophoresis.

Purification with exonuclease and alkaline phosphatase

N. gonorrhoeae porB and *tbpB* PCR products were purified using Exosap IT purification kit (Biomedica Hungária Ltd., Budapest). The sample was heated at 37 ° C for 45 minutes, at 80 ° C for 15 minutes, and then incubated at 4 ° C.

Big Dye

We used the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) and the same forward and revers primers as for the *porB* and *tbpB* PCR methods. Fluorescently labeled dideoxynucleotides were included in the BigDye® Terminator v3.1 Cycle Sequencing Kit. The study was conducted in accordance with international research (Martin IM, 2004) as previously described.

NucleoSEQ Column PCR Purification

Last purification was made by NucleoSEQ Column PCR Purification Kit (Macherey-Nagel, Germany), with gel-filtration technique. After we added the final product to formamide and placed it in PCR at 95 ° C for 5 minutes. The final product was then forwarded to Biomi Ltd. (Gödöllő) for the analysis of sequences.

Sequence analysis with ChromasLite

The sequences were analysied with the programme CromasLite 2.1., and than were assigned using the NG-MAST website.

Construction of Phylogenic tree

The aim of phylogenetic studies is to deduce evolutionary relationships. To compare the sequences, it is necessary to arrange the homologous nucleotide positions one after the

other, which is the alignment process. The next step in the phylogenetic reconstruction is the designation of the phylogenetic tree constructing algorithm.

MEGA6 was used to analyze azithromycin-resistant sequence types in 2014. Phylogenetic analysis was performed by a neighbour-joining statistical method with a maximum composite likelihood model. The degree of similarity was determined using the highly similar sequence (Megablast) BLASTN Program of the National Library of Medicine of the National Center for Biotechnology Information.

Results

Epidemiological data between 2010 and 2014

The proportion of N.gonorrhoeae positive cultures

Over the period of the four years of the study the number and proportion of N. gonorrhoeae positive cultures from the STD center increased. 11% increase in 2011, 28% increase in 2012, 18% increase in 2013 and 4% increase in 2014 was observed, respectively. The ratio of gonorrhea positive patients to the number of samples collected also increased from year to year, which supports the spread of gonorrhea infection.

The male to female ratio of patients

Over the period of 5 years, 85.4% of cases examined were subsequently determined to be positive for *N. gonorrhoeae* were men, with the balance of cases female (14.6%). 74% of the male patients are between 20 and 40 year old, while in women it is approximately 67%.

Distribution of infection according to anatomical site based on culture

Between 2010 and 2013, 80% of the pozitive samples were from the urethra, 4% from the pharynx and 16% from the rectum. In women, 62% of the samples were from the cervix, 20% from the urethra and 8%-8% from the anus and pharynx. In women, urethral and pharyngeal infection often were identified in parallel, besides anorectal or pharyngeal infections could occur. In special cases, conjunctiva infection was also observed, which can be caused by autoinoculation of genital secretion.

Urethritis was found in 77.3 % of male patients, while in females the dominant anatomical site of infection was the urethra (68.9 %) and cervix (65.5 %). Symptomatic infections or asymptomatic carrier states were detected in the anus (20.2 %/44.8 %) and in the pharynx (17.9 %/24.1 %) in male/female patients, respectively.

Pharyngeal and rectal infections are the most important concerning the spread of resistance, since pharyngeal and rectal gonorrhoea is mostly asymptomatic meaning that commensal *Neisseria spp.* and *N.gonorrhoeae* can co-exist for extended time periods in the pharynx and share genetic material. This is likely to include the transfer of antibiotic resistance genes and therefore the development of antibiotic resistance in *N. gonorrhoeae*. Besides, some antibiotics, such as cefixime, spectinomycin, do not reach enough

concentration in the pharynx, and due to asymptomatic infection, the individual may become an asymptomatic carrier.

Antimicrobial susceptibility in the last 5 years

The prevalence of penicillin and tetracycline resistance varied from 70 to 80% year to year. Ciprofloxacin resistance ranged from 60 to 70% between 2010 and 2013 and decreased to 40% by 2014. Data suggest that penicillin, ciprofloxacin, tertracycline can not be used for empirical treatment. Resistance to cefixime increased to 1.6% in 2015. All isolates were susceptible to spectinomycin and ceftriaxone. However, the use of spectinomycin is limited by the rapid development of spectinomycin resistance, and decreased efficacy of pharynx infection.

Analysis of azithromycin-resistant strains in 2014

30.0 % of all the strains- were resistant to azithromycin, according to the breakpoints of EUCAST. The percentage of azithromycin resistance showed a significant increase from 15.9 % in 2013 to 30.0 % in 2014 ($\chi 2 = 11.4437$, P value is 0.000717,$P < 0.001$). The ratio of female/male patients was 1: 7,3, which is slightly different from the whole patient population (1:5,6). The median age was 33.6 years. MICs of ≥1 mg/L for azithromycin were observed in 7.0 % of all gonococcal strains.

Our theory for the increasing azithromycin resistance is that, according to ESAC, azithromycin has become the second most commonly used antimicrobial in Hungary. Hence, patients might have been treated previously with azithromycin for an infection, microbiome in the pharynx or anus, could have been exposed to this antibiotic several times, could have acquired resistance and acted as reservoirs of genes. According to that WHO recommended that an antimicrobial should not be used when >5 % of the strains are resistant, the use of azithromycin should be considered further. Besides, there are some studies which do not report in vitro synergy between third-generation cephalosporins and azithromycin. The clinical efficacy of the dual therapy with ceftriaxone and azithromycin could be lower than ceftriaxone monotherapy since the combination was less bactericidal as ceftriaxone alone in time-kill experiments.

***Chlamydia trachomatis* infection**

In 2014, in our laboratory, 6.7 % of the samples of the STD center were Chlamydia-positive and 12.2 % were gonorrhoea-positive by multiplex RT-PCR. Only 14.7 % of the gonorrhoea-positive samples were co-infected with *C. trachomatis*.

Results of molecular typing

52 strains studied by NG-MAST method in 2013

Based on NG-MAST, the 52 isolates were subdivided into 22 different STs. The eight new STs, which had not been previously described in other parts of the world, were assigned as ST 10081 to ST 10088 using the NG-MAST website. The majority (61.5%) of the strains belongs to four frequently isolated STs; ST 2992 was represented by 10 (19.2%) isolates, ST 1407 and ST 4995 by 9 (17.3%) isolates each and ST 225 by 4 (7.7%) isolates. ST 8517 and ST 359 were present in 2 (3.8%) isolates each.

The most prevalent strain in Hungary, ST 2992, was the second most common strain according to Euro-GASP's 2010 data (Chisholm SA, 2013). 90% was resistant to tetracycline, 40% was resistant to azithromycin while all strains were susceptible to ciprofloxacin and isolates had low cefixim and ceftriaxon MIC.

Based on the data of Euro-GASP, multi-resistant ST1407 reached 16% in 2010 in Europe. While 17.3% was observed in 2013, in Hungary. Euro-GASP has reported that this is the strain which causes most of the therapeutic failures and is associated with ciprofloxacin resistance, decreased susceptibility to cefixim and elevated azithromycin and ceftriaxone MIC. In our study 56% of the strains, belonging to ST 1407, were resistant to azithromycin. 22% were moderately susceptible to azithromycin. 100% were resistant to tetracycline and ciprofloxacin. One of them was resistant to cefixim. 67% showed decreased susceptibility to cefixime (MIC≥0,025mg / l), while 89% to ceftriaxone (MIC≥0,012mg / l). As described in France, this is the sequence type that only develops a high level of resistance on the penA gene, cefixim and ceftriaxone, with only one further mutation.

Based on our studies, the third most common sequence type was ST 4995. It showed resistance to ciprofloxacin, tetracycline, and 22% of the strains had elevated azithromycin MIC. While we did not find elevated cephixime and ceftriaxone MICs. The relative high incidence of ST4995 is unique; no international publication about the importance of ST4995 was found.

The fourth most common sequence type in Hungary was ST 225 with 7.3%. According to Euro-GASP, ST 225 was the third most common in Europe and it was associated with ciprofloxacin-resistance. In our study, 100% of ST 225 were tetracycline-resistant and

50% were azithromycin-resistant. Moreover, their cefixime and ceftriaxone MICs were also increased.

Increased cefixime MICs (> 0.025mg / l) belonged to ST 1407, ST 225 and ST 2212, while higher ceftriaxone MICs (≥ 0.012mg / l) belonged to ST 1407, ST 225, ST 2569, ST 2212 and ST 210. High-level ciprofloxacin-resistance (MIC over 32mg/l) was associated with ST 225 and ST 1407. 98% of the isolates of ST 4995 were high-level tetracycline-resistant (MIC≥16 mg/l) characterisctic of plasmid-mediated resistance.
According to the European guideline, the first choice treatment is 1x500 mg ceftriaxone im. combined with 2 g oral azithromycin. However, the combination therapy might not be appropriate treatment in the case of Hungary, as 60% of the four most common sequence types are resistant to azithromycin.

50 azithromycin-resistant sequence types in 2014

The 50 *N. gonorrhoeae* resistant or intermediate-resistant isolates to azithromycin were divided into 34 NG-MAST sequence types, and a unique NG-MAST sequence type was found for 10 isolates. The three dominant strains were ST1407, ST4995 and ST11064, each represented by 5 isolates (10–10 %). ST 1407 had the highest levels of azithromycin MICs. ST 4995 possessed high level of tetracycline and ciprofloxacin resistance. This ST retained its leading role among the Hungarian strains. The frequent occurrence of ST 4995 suggests that this sequence type may be successful and may cause therapeutic failure in the future. ST 11064 has not been mentioned so far. It is a reasently spreading strain that was not identified in the previous year's study. All ST 11064 was susceptible to cefixime, ciprofloxacin. ST 1407 and 11064 had low-level tetracycline resistance which suggests that it is a chromosomally mediated mutation.

Phylogenetic studies

In case of STs in 2014

According to the constructed phylogenic tree, the azithromycin-resistant and intermediately resistant strains, isolated in 2014, were divided into three major groups based on closer relationship. From the most prevalent STs, the first group contained ST4417; ST1407 belonged to the second group, whereas the third group contained ST4995 and ST11064. The biggest similarity-99 %- was detected between ST21, ST11064 and ST11703. Based on the exact definition of genogroup—one identical allele is shared and the other allele shows a similarity of ≥99 % — ST21, ST11064 and ST11703

make up a genogroup. This genogroup is named G11064, since ST11064 is the predominant ST within the group. ST 1407, ST 8826 and ST 3378 also make up a genogroup (G 1407). As the latter strains are azithromycin resistant and por 908 allele build them up, it can be considered as a successful allele in spreading azithromycin resistance.

STs isolated abroad and in Hungary

According to the high diversity of *porB* alleles, we compared the *porB* alleles of azithromycin-resistant strains isolated in our laboratory and from Japan, Canada, Italy. Based on the phylogenetic tree, por 908 and the related alleles have been successfully contribute to the spread of azithromycin resistance all over the world.

Conclusions

The study of the *Neisseria gonorrhoeae* strains identified by culture has made it possible to supplement the international results with new Hungarian data. Our results are partly similar to West European data, but they also provide new data:

1. Our results represent the proportion of positive *N.gonorrhoeae* cultures (in our microbiology laboratory), the anatomical appearance of infection, the distribution of infection according to gender and age, and the antimicrobial resistance.
2. We have drawn attention to the need for antibiotic susceptibility testing in the diagnostic pathway by the increased resistance of first choice antibiotics.
3. We have succeeded in introducing nucleotide sequence-based typing methods (NG-MAST) of N. gonorrhoeae in our laboratory.
4. This is the first study in our country that describes NG-MAST sequence types and their resistance phenotypes.
5. 18 sequence types, which had not been previously dscribed, were assigned on ng-mast website.
6. Based on the appearance of the different sequence types, some of the common sequence types are identical to the Western European STs (ST 1407, ST 225, ST 2992). However, there are different, dominant sequences in our country (ST 4995, ST 11064), which are not mentioned in international studies.

   Phylogenetic studies have drawn attention to successful azithromycin-resistant genotype genomes.
7. Successful sequence types and knowledge of their resistance data can provide a basis for developing appropriate regional empirical therapeutic recommendations. In 2013, 60% of the 4 most common ST strains were resistant to azithromycin. It appears that the combination of ceftriaxone and azithromycin may not be appropriate therapeutic recommendation in case of Hungary. To avoid the spread of further azithromycin resistance, the use of azithromycin should be restricted to cases when its usage is definitely necessary, like cases co-infected with chlamydia or patients allergic to cephalosporins.

Bibliography of the candidate's publications

List of publications related to the thesis

1. Brunner A, Nemes-Nikodem E, Mihalik N, Marschalko M, Karpati S and Ostorhazi E. (2014) Incidence and antimicrobial susceptibility of Neisseria gonorrhoeae isolates from patients attending the national Neisseria gonorrhoeae reference laboratory of Hungary. BMC Infect Dis, 14:433.

IF: 2,613

2. Nemes-Nikodém E, Brunner A, Pintér D, Mihalik N, Lengyel Gy, Marschalkó M, Kárpáti S, Szabó D, Ostorházi E. (2014) Antimicrobial Susceptibility And Genotyping Analysis Of Hungarian *Neisseria Gonorrhoeae* Strains In 2013. Acta Microbiol Immunol Hung, 61 (4): 435–445.

IF: 0,778

3. Brunner A, Nemes-Nikodem E, Jeney Cs, Szabo D, Marschalko M, Karpati S, Ostorhazi E. (2016) Emerging azithromycin-resistance among the Neisseria gonorrhoeae strains isolated in Hungary. Ann Clin Microbiol Antimicrob. 15(1):53.

IF: 2,376

4. Nemes-Nikodém E, Brunner A, Tóth B., Tóth V., Bánvölgyi A., Ostorházi E. (2015) *Neisseria gonorrhoeae* törzsek rezisztencia adatai Magyarországon, Orv. Hetil., 156(1): 25–28.

IF: 0,291