

ANTIMICROBIAL SUSCEPTIBILITY AND GENOTYPING ANALYSIS OF HUNGARIAN *NEISSERIA GONORRHOEAE* STRAINS IN 2013

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Purpose: Emergence and spread of antimicrobial resistance in *Neisseria gonorrhoeae* is a major public health concern worldwide. The current study aims to determine the antimicrobial resistance in *N. gonorrhoeae* and associated molecular typing to enhance gonococcal antimicrobial surveillance in Hungary. **Methods:** In the National *N. gonorrhoeae* Reference Laboratory of Hungary 187 *N. gonorrhoeae* infections were detected in 2013, antibiograms were determined for all the isolated strains, and 52 (one index strain from every sexually contact related group) of them were also analyzed by the *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) method. **Results:** Twenty-two different NG-MAST sequence types (STs) were identified, of which 8 STs had not been previously described. In Hungary, the highly diversified gonococcal population displayed high resistance to penicilline, ciprofloxacin and tetracycline (the antimicrobials previously recommended for gonorrhoea treatment). Resistance to the currently recommended extended spectrum cephalosporins were rare: only two of the expected strains, an ST 1407 and an ST 210, had cefixime MIC above the resistance breakpoint. By the revision of our National Treatment Guideline, it must be considered, that the azithromycin resistance is about 60% among the four most frequently isolated STs in Hungary.

Keywords: *Neisseria gonorrhoeae*, sequence typing, antimicrobial susceptibility, Hungary

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Introduction

Over the last few years, the number of gonorrhoea cases has increased regularly in Europe [1]. The need for effective treatment and control of gonococcal infections are evident, given the serious complications of gonorrhoea, such as epididymitis, pelvic inflammatory disease, infertility, ectopic pregnancy, early abortion and perinatal infections, furthermore a fivefold increased risk for human immunodeficiency virus (HIV) acquisition and transmission [2]. Knowledge of local susceptibility patterns combined with incidence of various molecular types of *N. gonorrhoeae* is essential to establish the best regional treatment guideline. Among the currently available sequence-based methods *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) is one of the most advantageous, because it generates a simple numerical sequence type based on the combined sequences of two genes (*porB* and *thpB*). In addition, an internationally accessible web database allows strain comparison worldwide [3]. Several studies have applied NG-MAST to characterise gonococci exhibiting resistance to the third generation cephalosporins [4–6], and the European Gonococcal Antimicrobial Resistance Surveillance Programme (EURO-GASP) made the first study to supplement a regional antimicrobial resistance surveillance programme with molecular typing. This Programme provided novel information on NG-MAST STs circulating in 21 European countries and also provided insight into the dissemination of antimicrobial resistant gonococcal clones within Europe [7]. As Hungary did not participate in EURO-GASP in 2009, only very low numbers (17) of *N. gonorrhoeae* were available from 2010 for inclusion. Our recent work complements the European database with the results of molecular epidemiological surveillance of consecutive isolates of all antimicrobial phenotypes in Hungary in 2013. The presence of regional surveillance data about antimicrobial resistance of *N. gonorrhoeae* is a good approach to estimate the global burden of resistance of this microorganism as well as to establish the strategies to apply the best empirical treatment

Materials and Methods

Bacterial isolates

From January to December 2013 *N. gonorrhoeae* strains were obtained from patients in the National *Neisseria gonorrhoeae* Reference Laboratory at the Centre of Sexually Transmitted Diseases, Department of Dermatology, Venereology and Dermatooncology of Semmelweis University Budapest, Hungary. The

isolates were cultured and stored from consecutive symptomatic gonorrhoea patients and from their asymptomatic contacts. All isolates were collected as part of standard patient care and patients gave and signed informed consent. Patient data was analysed according to law 1997/CLIV 26§ taking into account maximum privacy rights and anonymity of patients (Parlament of Hungary, 1997). The Semmelweis University Regional and Institutional Committee of Science and Research Ethics have approved the analysis (TUKÉB: 161/2014). For the identification of *N. gonorrhoeae* cervical, anal, urethral and pharyngeal swabs were taken and cultured on preheated VCA3 agar (Biomérieux, Hungary). Cultures were incubated for 48 hours at a temperature of 36.5 °C with 5% carbon dioxide, and their identification and penicillinase production was confirmed by NH-API strip tests (Biomérieux, Hungary). The selection criteria for genotyping analysis were the follows: we used only one isolate from one patient, and only isolates from the index patients were included for genotyping analysis. Contacts from the index case were excluded.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC; mg/l) of cefixime, ceftriaxone, penicillin, tetracycline, azithromycin, spectinomycin and ciprofloxacin were determined on PVX chocolate agar (Biomérieux, Hungary) using MIC strip tests (Liofilchem, Italy), according to the manufacturer's instructions, using a direct colony suspension equivalent to a 0.5 McFarland standard. Testing conditions included incubation at 36.5 °C, 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) [5]. The isolates were classified as having a presumed high level resistance to tetracycline (TRNG) if MIC was ≥ 16 µg/mL, and as having a presumed plasmid-mediated resistance to penicillin (PPNG) if they were β -lactamase positive [6].

Isolation of genomic DNA

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

gonococcal subculture was made in PCR water and DNA was isolated with GeneAll Ribospin extraction kit (Geneall Biotechnology, Korea).

Molecular epidemiological typing

N. gonorrhoeae multiantigen sequence typing (NG-MAST) was performed as previously described [6]. Briefly we determined the sequences of the 52 *N. gonorrhoeae* *porB* and *thpB* PCR products, after their preliminary purification by the Exosap IT purification kit (Affymetrix, USA) we used the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) and the same forward and revers primers as for the *porB* and *thpB* PCR methods. Last purification was made by NucleoSEQ Column PCR Purification Kit (Macherey-Nagel, Germany). Nucleotide sequences were determined by capillary electrophoresis, with 50 cm capillary and POP-7 polimer on ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

NG-MAST allel numbers of the more variable segments of *porB* and *thpB*, and STs including the new allels and STs were assigned using the NG-MAST website [3].

Results

From January to December 2013 a total of 222 *N. gonorrhoeae* strains were obtained from 187 patients, 29 patients had *N. gonorrhoeae* infection on more than one anatomical site. The number of the sexual contacts of the patients ranged from 1 to 12, with an average of 3.9 in a half year prior the gonorrhoea infection. During the contact research the 187 patients were tabulated in 52 sexually contacted network groups. Every network group contained only the patients who mutually and independently admitted to have sexual contact with a member of the group. The *N. gonorrhoeae* strains isolated from patients attending to the same network group all had a group specific antibiotic resistance pattern. The first symptomatic patient was selected as index patient from every network group. After selection 52 *N. gonorrhoeae* isolates from the index patients were examined according to the antibiotic susceptibility and molecular epidemiological typing.

Among the 52 patients 5 were female and 47 were male. More than half, 24 of the men were heterosexual, 2 were bisexual, and 21 declared that they were men who have sex with men (MSM). Ages of the examined patients ranged from

15 to 64 years. The dominant anatomical site of infection was urethra, 43 strains were isolated from urethra, 3 from cervix, 2 from pharynx and 4 from anus.

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. ST 10081 and ST 10082 have new *porB* alleles, *porB* 5921 and *porB* 5922 combined with the common *tbpB* alleles, *tbpB* 29 and *tbpB* 110, respectively. STs 10083 to 10088 contain known alleles in new combinations. The 14 other common STs were found in the NG-MAST database, they were described previously in other parts of the world as well. 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. All the other STs were detected in single (1.9%) isolates.

The frequency and antibiotic susceptibility patterns of the 22 STs are summarized in Table I. Only two isolates showed susceptibility to all tested antibiotics; these isolates attend to ST 387 and ST 9901. The other 50 isolates were resistant to at least one antibiotic.

Only 6/52 (11.5%) isolates were sensitive to penicillin, among the 46/52 (88.5%) penicillin resistant strains 7 (15%) were penicillinase producers. Forty-nine of the 52 strains (94%) were resistant to tetracycline and among the tetracycline resistant strains 9/49 (18.4%) were TRNG. Twenty-nine of the 52 strains (56%) were resistant to ciprofloxacin. All the 52 strains were sensitive to spectinomycin. The azithromycin MIC was above 0.5 mg/l in 20 strains (38%) and are declared as resistant to azithromycin according to the EUCAST [5] recommendation. This MIC was equal or lower as 0.25 mg/l in 27 (52%) of isolations, which were sensitive to azithromycin. Five strains had an elevated azithromycin MIC of 0.38 mg/l. Among the isolations representing the most common four STs (ST2992, ST1407, ST4995, ST225) 19/32 (60%) were not sensitive to azithromycin. All the isolates were sensitive to ceftriaxone, and only two isolates were resistant to cefixim from ST1407 and ST210. More than half of the strains had elevated ceftriaxone and cefixim MIC (0.023mg/L = ceftriaxone MIC = 0.064mg/L, 0.023mg/L = cefixim MIC=0.19mg/L) in ST1407, ST225 and ST210. The distribution of MIC values of the different STs is summarized in Table II.

Table I

Incidence and antimicrobial susceptibility of NG-MAST STs detected in Hungary in 2013.
S = Sensitive, R = resistant, I = elevated MIC

NG-MAST ST	Incidence (%)	Antimicrobial-susceptibility						
		Penicillin	Ciprofloxacin	Tetracycline	Spectinomycin	Azithromycin	Cefixime	Ceftriaxone
2992	19.2	20% S 80% R	S	10% S, 90% R	S	30% S, 30% I, 40 % R	S	S
1407	17.3	100% R	R	R	S	22% S, 22% I, 56% R	89% S, 11% R	S
4995	17.3	11% S 89 %	R	R	S	78% S, 22% R	S	S
225	7.7	100% R	R	R	S	50% S, 50% R	S	S
359	3.8	S	S	R	S	S	S	S
8517	3.8	R	S	R	S	S	S	S
210	1.9	R	R	R	S	S	R	S
387	1.9	S	S	S	S	S	S	S
995	1.9	R	S	R	S	S	S	S
2212	1.9	R	R	R	S	S	S	S
2569	1.9	R	R	R	S	R	S	S
6155	1.9	R	S	R	S	S	S	S
8343	1.9	R	R	R	S	S	S	S
9901	1.9	S	S	S	S	S	S	S
10081	1.9	R	R	R	S	R	S	S
10082	1.9	R	R	R	S	S	S	S
10083	1.9	R	R	R	S	S	S	S
10084	1.9	R	S	R	S	S	S	S
10085	1.9	R	S	R	S	S	S	S
10086	1.9	R	S	R	S	R	S	S
10087	1.9	R	S	R	S	R	S	S
10088	1.9	R	S	R	S	R	S	S

Discussion

Surveillance programs monitoring the antimicrobial susceptibility and molecular characteristics of *N. gonorrhoeae* are essential to implement adequate empirical treatment guidelines. The last national guideline on the diagnosis and treatment of gonorrhoea [11] was published in Hungary in 2002. Due to this guideline 1 × 250 mg ceftriaxone i.m. or 400 mg ofloxacin per os is recommended as first

Table II

Distribution of MIC of cefixime and ceftriaxon among the different *Neisseria gonorrhoeae* STs isolated in Hungary in 2013

The lowest detectable cefixim (CFM) and ceftriaxone (CRO) MICs are 0.016 and 0.002 mg/l, respectively.

ST	Anti- Num-ber of isolates with cefixim and ceftriaxon MIC (mg/L)											
	microbial	≤0.002	0.012	≤0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	
2992	CFM			10								
	CRO	10										
1407	CFM											
	CRO	1	2	1	2	1	3	1	1	1		
4995	CFM			9								
	CRO	9										
225	CFM			1								
	CRO			3								
Other STs	CFM			17								
	CRO	15	4	1								1 (ST210)
		1 (ST210)										

choice, or 400 mg cefixim per os or 500 mg ciprofloxacin per os can be administered in the cases of uncomplicated gonorrhoeal urethritis or cervicitis. In Hungary, gonorrhoeal infection cases must be notified to the National Epidemiological Center regardless of whether they were detected with nucleic acid amplification tests (NAATs), culture or only microscopically. Publication about Hungarian gonococcal incidence data is available only in Hungarian in the Report of the National Epidemiological Center, but neither antibiotic resistance nor molecular typing results are included [12]. About 14% (222/1563) of the notifiable infections were diagnosed at the STD Center of Semmelweis University during this period, and the distribution according to gender, age and location of the symptoms was the same as the national results. Among our index patients men was much more than women, it may simply indicate the likely order in which cases were identified, men are more likely to be symptomatic. The National Reference Laboratory of *N. gonorrhoeae*, as a part of the STD Center of Semmelweis University, receives patients from all over the country. At our centre, diagnosis of gonorrhoea is made by culture, meaning that AMR development within Hungary can be monitored retrospectively.

The EURO-GASP demonstrated antibiotic resistance and molecular characterization results but only about 17 *N. gonorrhoeae* isolations collected in 2010 in Hungary [7]. The present study describes the first internationally reported gonococcal AMR data together with the first molecular characterization of

N. gonorrhoeae isolates in Hungary. This study shows the antimicrobial susceptibility and the NG-MAST results of *N. gonorrhoeae* single strains, isolated from 52 patients assumed that they were not linked to each other. The 52 isolates were subdivided into 22 different STs, but more than 60% of the highly diverse bacterium population belonged to 4 STs, ST2992, ST1407, ST4995 and ST225, previously described in other parts of the world [3]. The EURO-GASP study [7] pointed out that 12 of the 17 isolations collected from Hungary in 2010 belonged to the genogroup1407 (10 strains to ST5332 and 2 strains to ST1407), but among these isolates neither ST2992, nor ST4995 nor ST225 were found. This discrepancy may be explained by the facts that very low numbers of isolates were available for inclusion in the EURO-GASP study, or that our isolates were collected 3 years later, in 2013. In 2010 ST4995 was not described, but our other 3 frequent STs were the most frequent STs in EURO-GASP as well. Between 2010 and 2012 G1407 was currently the most frequently detected genogroup worldwide from Canada to Japan [13, 14], in Europe 23% of the isolates belonged to G1407, 10% to G225 and 8% o isolate belonged to G2992 [15]. In Hungary in 2013 the most frequently isolated ST was ST2992 (19%) followed by ST1407 and ST4995 (in 17% each), and ST225 was isolated in 7%. The relative high incidence of ST4995 is unique; no international publication about the importance of ST4995 was found.

In addition from a personal communication we know that nineteen of 1445 (1.3%) typed gonorrhoea episodes in Scotland in 2013 were ST4995. All Scottish patients identified with ST4995 were male, suggesting circulation in networks of men who have sex with men, although sexual orientation data was not available. The isolates from Scotland were resistant to ciprofloxacin (MICs ranged from 3–12 mg/L by Etest) and had high-level tetracycline resistance characteristic of plasmid-mediated resistance. Seven of 12 isolates were PPNG. Isolates were susceptible to ceftriaxone, cefixime, spectinomycin and azithromycin by agar dilution [Kirstine Eastick, Director of Scottish Bacterial STI Reference Laboratory personal communication].

Among our isolates high prevalence of resistance was observed for previously recommended antimicrobials such as tetracycline (94%), ciprofloxacin (56%) or penicillin (88.5%), in accordance with other international data [7, 13–18]. These results clearly show that the use of these three antibiotics for the empirical treatment of gonococcal infections is no longer safe. The use of antibiotic treatments without performing sensitivity tests generates treatment failures [19–21] even with the antibiotics currently proposed as first choice antibiotics. The extensive use of antibiotics also leads to a positive selective pressure for specific international multidrug resistant (MDR) clones such as G1407, or for resis-

tance against an often used antibiotic, as we found only 52% azithromycin sensitivity among our isolates. G1407 isolates circulating currently in Europe are ciprofloxacin resistant, strongly associated with decreased susceptibility or resistance to cefixime and show raised MICs for ceftriaxone and azithromycin [7]. Despite the fact that no ceftriaxone resistant isolates were identified in our study, the proportion of isolates with a ceftriaxone MIC = 0.016 mg/L was significantly lower among our ST1407 and ST225 strains than in other STs. Furthermore, among our ST1407 and ST225 isolates the proportion of strains with a cefixime MIC = 0.032 mg/L was also decreased in contrast with other STs. Isolates with an extended spectrum cephalosporine (ESC) MIC of 0.125 mg/L have resulted in treatment failures with ESCs [22,23]. Accordingly, we considered an ST1407 isolate as having at least a decreased susceptibility to cefixime. Furthermore, we found an ST210 isolate with a cefixime MIC of 0.19 mg/L associated with a ceftriaxone MIC of 0.064 mg/L, therefore this ST might have a great chance to become a successfully selected MDR clone. Spectinomycin remains an antibiotic still effective in treatment against MDR *N. gonorrhoeae* strains. However, adoption of spectinomycin as the routinely used drug of choice was soon followed by reports of spectinomycin resistance [24]. All our isolates regardless of represented STs were sensitive to spectinomycin in consistence with the results of other authors [7, 16–18].

This is the first study describing antimicrobial resistance and NG-MAST clonality of gonococcal isolates detected in Hungary. *N. gonorrhoeae* strains isolated in Hungary in 2013 showed a high genetic diversity but also showed a dominance of 4 internationally known clones. Due to the spread of these MDR clones the antibiotic susceptibility of our isolates is decreased, meaning either resistance to the worldwide previously recommended antibiotics as penicillin, ciprofloxacin or tetracycline, or elevated MICs of the recently recommended therapeutic options such as ESCs or azithromycin. In this context, it is essential to increase numbers of cultures in gonococcal infections in Hungary to study and monitor resistance patterns and molecular clonality. We emphasize the urgent need for promptly revision of the National Treatment Guideline of gonorrhoea in Hungary; the present study provides an evidence base for this recommendation. The European Guideline recommends 500 mg ceftriaxon combined with 2 g azithromycin as first choice therapy, but by the revision of our National Treatment Guideline, it must be considered, that the azithromycin resistance is about 60% among the four most frequently isolated STs in Hungary.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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