Original paper

Plasmid-mediated resistance to tetracyclines among *Neisseria gonorrhoeae* strains isolated in Poland between 2012 and 2013

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Abstract

Introduction: One of two main mechanisms of resistance in tetracycline-resistant *Neisseria gonorrhoeae* (TRNG) is associated with the presence of TetM protein responsible for actively blocking of the tetracycline target site in the 30S ribosomal subunit. This mechanism is encoded by conjugative plasmids. The second mechanism is chromosomal in nature and due to mutations in specific genes.

Aim: To determine the incidence and type of *tetM* determinants in TRNG strains isolated from patients presenting with gonorrhea infection to the Dermatology and Venereology Clinic in Warsaw in 2012–2013.

Material and methods: Tetracycline and doxycycline susceptibility was determined by E-Tests. The presence and type of the *tetM* gene were determined by polymerase chain reaction.

Results: Tetracycline resistance was detected in 50.8% of the evaluated strains. The TRNG strains containing the *tetM* plasmid constituted 13.8% of all the evaluated strains. Dutch type *tetM* constituted 12.3% and American type *tetM* 1.5% of all the evaluated strains. In the remaining TRNG strains, resistance to tetracyclines was presumably chromosome-encoded. The minimal inhibitory concentration (MIC) of tetracycline ranged from 0.25 to 32.0 mg/l, MIC_{50} = 2.0 mg/l, MIC_{90} = 32.0 mg/l. The MIC of doxycycline ranged from 0.25 to 32.0 mg/l, MIC_{50} = 4.0 mg/l, MIC_{90} = 16.0 mg/l.

Conclusions: Unlike most of European countries, in 2012–2013 in Poland, the Dutch type *tetM* was found to be much more common than the American type. Minimal inhibitory concentration values of tetracycline and doxycycline were similar, with doxycycline exhibiting a somewhat lower effectiveness *in vitro* than tetracycline towards chromosome-mediated tetracycline resistant strains of *N. gonorrhoeae*.

Key words: *Neisseria gonorrhoeae, tetM* Dutch type, *tetM* American type, tetracycline resistance.

Introduction

Tetracyclines are wide-spectrum antibiotics inhibiting bacterial protein synthesis via their effect on the 30S ribosomal subunit. Tetracycline resistance in different bacterial species, both Gram-negative and Gram-positive, is most commonly determined by a large group of efflux proteins, e.g. TetA to TetL belonging to the major facilitator superfamily (MFS) and by another group of proteins, such as TetM to TetW, responsible for actively blocking of the tetracycline target site in the 30S ribosomal subunit [1, 2]. Apart from special proteins, antibiotic resistance may be a result of a mutation in porin and transmembrane pump regulator genes. Tetracycline resistance of *Neisseria gonorrhoeae* may be determined either by plasmid-encoded TetM protein or by mutations in chromosomal genes. The presence of the plasmid-encoded TetM protein typically determines the MIC of tetracycline (16–64 mg/l). Three types of conjugative plasmids have been identified in *N. gonorrhoeae*. Two of them, both of 25.2 MDa, contain the *tetM* gene. The third one, of 24.5 MDa, does not encode antibiotic resistance. Restriction mapping and Southern blot techniques helped to detect differences between these plasmids; consequently, the Dutch and American plasmid types were identified [3, 4]. The *tetM* region of both plasmid types was

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sequenced and the discovered differences served as the basis for calling the relevant *tetM* determinants: Dutch and American. Currently, we know that typically, though not always, the plasmid type is consistent with the *tetM* determinant type [5].

Tetracycline resistance in epidemic *N. gonorrhoeae* strains (e.g. G1407 genogroup) is often a result of mutations in *penB* (encoding porin B) [6–9], *penC* (encoding PilQ secretin) [6, 8, 10] and overproduction of the MtrCDE efflux pump, associated with mutations in the *mtrR, mtrC* promotor regions and in the *mtrR* gene [9, 11, 12]. Chromosomal mutations cause an increase in the minimal inhibitory concentration (MIC) of tetracycline, usually up to 2–4 mg/l. A comparable level of resistance is also provided by much more rare mutations in the *rpsJ* gene encoding the S10 ribosomal protein [13]. As a result of increasing resistance of *N. gonorrhoeae* to tetracyclines these antibiotics are not used in monotherapy to treat *N. gonorrhoeae* infections in Europe. However, either tetracycline or doxycycline may be used instead of azithromycin in combination with third-generation cephalosporin, especially in mixed infections, e.g. gonorrhea and chlamydia [14–17].

Aim

The purpose of our work was to determine the incidence and type of *tetM* determinants in TRNG strains isolated from patients presenting with gonorrhea infection to the Dermatology and Venereology Clinic in Warsaw in 2012–2013.

Material and methods

Bacterial strains

A total of 65 *N. gonorrhoeae* isolates were evaluated. Nine strains were isolated from women, 56 strains were obtained from men. They had been obtained from urethral (54 strains), cervical (9 strains) or pharyngeal (2 strains) swabs of patients who visited the outpatient facility at the Dermatology and Venereology Clinic, Medical University in Warsaw at the end of 2012 and throughout 2013. Out of the strains isolated in 2012, we selected those previously unreported [18]. The strains were identified to the species level based on their colony and cell morphology as well as oxidase test and carbohydrate fermentation test results (API NH, bioMérieux). Reference *N. gonorrhoeae* strains ATCC® 49226™, ATCC® 31426™ and ATCC® 49981™ were included. Bacteria were stored at –70°C (Microbank, Fischer Scientific, USA).

Susceptibility testing

The isolated strains were tested for susceptibility to tetracycline and doxycycline using E-tests (bioMérieux, France). The tests were conducted in accordance with the manufacturer's instructions and current EUCAST guidelines. The tests were performed on plates with Mueller-Hinton Chocolate Agar enriched with BD IsoVitaleX™ (Becton Dickinson). E-tests were performed 10 min after inoculation and incubated in CO_2-rich atmosphere (Genbox, bioMerieux) at 37°C. Endpoint readings were conducted at 24 h, with the use of a magnifying glass. The results were interpreted according to EUCAST and CLSI guidelines [19, 20].

DNA isolation and PCR

DNA was isolated with the Genomic Mini kit (A&A Biotechnology, Poland), using the manufacturer-recommended reagents and procedure. C 1000™ Thermal Cycler (BIO-RAD) was used for polymerase-chain reactions (PCR). Each 50-µl sample contained 2 µl of analyzed DNA, 5 µl 10x buffer (MBI Fermentas), 5 µl 1.5 mM $MgCl_2$ (MBI Fermentas), 0.2 µl of Taq polymerase (1.25 U, MBI Fermentas), 2 µl 200 µM MdNTPS (MBI Fermentas), 2 µl 25 µM solution of each of the primers (oligo.pl, IBB PAN, Warsaw), and 32.8 µl of deionized H_2O. The following PCR protocol was used: 94°C, 3 min; 30 cycles at 94°C, 20 s annealing, 55°C, 60 s, 72°C, 90 s, 72°C, 7 min. We used previously reported primers [21]: UF 5'-CTCGAACAAGAGGAAGC-3'; AR 5'-GCATTCCACTTCCCAAC-3'; DR 5'-TGCAGCAGAGGGAGG -3'. Predicted PCR product sizes: UF+AR 778 bp (American type *tetM*), UF+DR 443 bp (Dutch type *tetM*). PCR products were visualized by 1% agarose gel electrophoresis (Bio-Rad) alongside GeneRuler 1kb DNA Ladder (Fermentas). Ethidium bromide-stained PCR products were visualized with Gel DOC ™ XR+ Imaging System (BIO-RAD).

Results

Thirty-three (50.8%) out of the 65 evaluated *N. gonorrhoeae* strains were found to be tetracycline resistant according to both EUCAST and CLSI criteria. Table 1 shows MIC value distribution and result interpretation according to EUCAST and CLSI. Table 2 presents the MIC_{50} and MIC_{90} values of tetracycline and doxycycline. In light of no existing criteria for interpretation, susceptibility testing results for doxycycline are not presented here. Tetracycline-resistant strains were mainly (72.8%) those with tetracycline MIC of 2–4 mg/l consistent with chromosome-mediated resistance, whereas strains with tetracycline MIC of 16–32 mg/l consistent with the plasmid *tetM* determinant constituted 21.2% of TRNG strains. We also assessed the presence and type of the plasmid *tetM* determinant. The results are shown in Table 3. The plasmid *tetM* determinant was found in 9 out of 33 TRNG strains (27.3%), which constituted 13.8% of all evaluated *N. gonorrhoeae* strains. Out of the 9 strains with the *tetM* determinant, 8 (88.9%) were of the Dutch type, and only 1 (11.1%) exhibited the American-type determinant. None of the evaluated strains exhibited either two different

Table 1. Tetracycline MIC values determined for 65 evaluated *N. gonorrhoeae* strains

Concentration [mg/l]	Resistance type	Tetracycline MIC *N* (%) of strains	Doxycycline MIC* *N* (%) of strains	Tetracycline-susceptibility interpretation	
				EUCAST	CLSI
0.25		1 (1.5)	2 (3.1)	S	S
0.5		5 (7.7)	5 (7.7)	S	I
1.0		26 (40.0)	16 (24.6)	I	I
2.0	Chromosomal	20 (30.8)	10 (15.4)	R	R
4.0	Chromosomal	4 (6.1)	23 (35.4)	R	R
8.0	Chromosomal	0 (0)	1 (1.5)	R	R
16.0	*tetM*	2 (3.1)	7 (10.8)	R	R
32.0	*tetM*	7 (10.8)	1 (1.5)	R	R

*MIC – minimal inhibitory concentration, CLSI – Clinical and Laboratory Standards Institute, EUCAST – European Committee on Antimicrobial Susceptibility Testing, S – susceptible, I – intermediate, R – resistant, *no breakpoints.*

Table 2. Tetracycline and doxycycline MIC_{50} and MIC_{90} for *N. gonorrhoeae*

Antibiotic	MIC range [mg/l]	MIC_{50} [mg/l]	MIC_{90} [mg/l]	Criteria	*N* (%) of strains		
					S	I	R
Tetracycline	0.25–32.0	2.0	32.0	EUCAST	6 (9.2)	26 (40.0)	33 (50.8)
				CLSI	1 (1.5)	31 (47.7)	33 (50.8)
Doxycycline	0.25–32.0	4.0	16.0	*			

*MIC – minimal inhibitory concentration, CLSI – Clinical and Laboratory Standards Institute, EUCAST – European Committee on Antimicrobial Susceptibility Testing, S – susceptible, I – intermediate, R – resistant, *no breakpoints.*

tetM determinants or a *tetM* plasmid together with a penicillinase plasmid.

Discussion

The prevalence of tetracycline resistance in *N. gonorrhoeae* depends on the time period and the country of strain isolation. In 2013, in India there were 12% of tetracycline resistant strains [22]; in 2010–2012, in Sri Lanka, 16.3% [23]; in 2010–2011/2012/2013, in Belarus it was 36/35/40%, respectively [24]; in 2010–2011, in Germany the percentage was 41.3% [25]; similarly in 2010/2011/2012, in Poland resistant strains constituted 42.9/38/49%, respectively [18]; in 2010–2012, in Indonesia 100% strains were resistant to tetracycline [23]. Studies on the worldwide prevalence of Dutch and American type conjugative plasmids encoding the TetM protein in *N. gonorrhoeae* strains showed the Dutch type plasmids to be more prevalent in Asian countries such as Indonesia (100%), Philippines (100%), and Thailand (100%) in 1988–1995 [21], in China in 1999–2006 (99.2%) [26] and in 2011–2012 (96.2%) [27], in Bangladesh (98.7%) [28], in Brazil (76.5%), in Guyana (61.1%), in Trinidad (95.5%), in Saint Vincent (93.3%) [29, 30], whereas the American type plasmids were more prevalent in Europe in 1988–1995 (80.5%) [21], in the UK in 1988–1995 (81.8%) [21], in Italy in 2003–2005 (77.8%) [31], in several African countries in 1988–1995 (98.3%) [21], and in Jamaica in 1988–1995 (63%), with a total Caribbean prevalence of 64.3% [21]. Our study showed that TetM-synthesizing *N. gonorrhoeae* strains isolated in Poland in 2013 are more often of the *tetM* Dutch type (88.9%). Meanwhile, we observe an increase in the prevalence of the *tetM* determinant in different countries. For example, the prevalence of *tetM* determinant in *N. gonorrhoeae* strains in China increased 18-fold in 1999–2005 (from 1.8% in 1999 to 32.8% in 2006) [26]. In Poland the prevalence of *tetM* in *N. gonorrhoeae* strains was 17.9/17.4/6.1% in 2010/2011/2012, respectively.

Table 3. The *tetM* determinant type in *N. gonorrhoeae* strains

Strain	Tetracycline MIC	Doxycycline MIC	*tetM* type
1	16.0	8.0	Dutch
2	32.0	16.0	Dutch
3	32.0	16.0	Dutch
4	32.0	16.0	Dutch
5	16.0	16.0	American
6	32.0	16.0	Dutch
7	32.0	32.0	Dutch
8	32.0	16.0	Dutch
9	32.0	16.0	Dutch

In recent years, tetracycline-resistance of *N. gonorrhoeae* isolates in Poland has remained at the same high level, with approximately 1/3 of isolates exhibiting plasmid-mediated resistance. Unlike in many other European countries, in Poland the prevalence of the Dutch type of *tetM* was much higher than that of the American type in 2012–2013. The reason of the observed difference is unknown. It does not seem to come from neighboring eastern countries either, because although the percentage of Dutch and American type of *tetM* in Russia was not investigated, *tetM* accounts only for 3% of *N. gonorrhoeae* tetracycline resistance in this country [32]. We have no data about *tetM* prevalence in Belarus and Ukraine, however the percentage of resistant strains in Belarus was about 40 [24]. The observed pattern of tetracycline resistance seems to be regional to Poland. The comparison with NG-MAST types of the previously isolated strains (data published before) [18] revealed that the *tetM* determinant was most often found in NG-MAST sequence type (ST) 1405. The type of the *tet*M gene was not determined at that time. The ST 1405 was the most prevalent type in Poland in 2010, 2011 and at the beginning of 2012 it was still relatively common, although it was partially replaced by epidemic ST 1407. Unlike 1407 that prevails in many European countries, ST 1405 hardly ever occurs outside Poland.

The MIC values for tetracycline and doxycycline were comparable, with doxycycline showing a slightly lower activity *in vitro* against TRNG not possessing *tetM* determinant, with tetracycline-resistance most likely to be chromosome mediated.

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Conflict of interest

The authors declare no conflict of interest.

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