Analysis of the Contribution of Molecular Mechanisms into Formation of Gonoccocal Resistance to Tetracycline

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We applied complex genetic analysis for evaluation of tetracycline-resistance markers in 129 clinical strains of *Neisseria gonorrhoeae* from Central, Privolzhskii, and Siberian regions. For detection of mutations in *rpsJ* gene and *MtrRCDE* locus we first used minisequence reaction followed by identification of products by MALDI-TOF mass spectrometry. The incidence of detection of resistance markers among the analyzed strains were: *tetM* — 3.1%, mutations in genes *rpsJ* — 82.2 %, *penB* — 62.8%, and *mtrR* — 54.3%. The analyzed genetic markers were not detected in 17.5% strains. *tetM* gene was detected in only 12.5% strains from the Central Region. No differences were revealed in regional distribution of other genotypes. Genotypes $tetM_{pres}$, $rpsJ_{mut}$, $mtrR_{mut}$, and $rpsJ_{mut}$, $penB_{mut}$, $mtrR_{mut}$ reliably predict tetracycline resistance. Microbiological and genetic testing of tetracycline resistance yielded similar results.

Key Words: *N.gonorrhoeae, resistance; tetracycline; minisequencing, mass-spectrometry*

Gonorrhea is still a prevalent diseases in the world. According to WHO data, 1.5 mln new cases were recorded in the North America, 1 mln in the West Europe, and 3.5 mln in the East Europe and Middle Asia, the world morbidity was 62 mln cases. In Russia, 100 cases per 100,000 persons [4,7] were recorded, therefore effective therapy of this infection is a pressing problem.

Tetracyclines (TC) are not currently recommended for the treatment of gonorrhea, but they are still used in Russia. The use of tetracyclines requires administration of multiple doses, which can reduce the efficiency of therapy and is fraught with overdose. The resistance to TC is still high and comparable to penicillin resistance. In Russian Federation 84.3% *N. gonorrhoeae* strains are resistant to TC [2].

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The resistance to TC in *N. gonorrhoeae* is mediated by both plasmid and chromosome mechanisms. High resistance is usually determined by the presence of *tet(M)*-determinant in a 25.2-MDa conjugative plasmid [11].

The following chromosome mechanisms of resistance are known: decreased permeability of internal structures of microbial cell (changes in porin proteins, *penB* mutation) [8], active efflux of the antibiotic (abnormal expression of MtrC-MtrD-MtrE-system proteins) [6], and changed affinity of ribosomal proteins to TC due to mutation in *rpsJ* gene [11]. These mechanisms can determine clinically significant gonococcal resistance to TC with minimum suppressing concentration (MSC) surpassing 4 μg/ml.

For instance, changes in Por-protein, in particular *penB* mutation in loop 3 of Por protein, G120D and A121D, are responsible for increased resistance to penicillin and TC [12]. Substitution for aspartic acid in positions 120 or 121 also conferred moderate antibiotic resistance. The substitution G120K

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conferred resistance comparable to that determined by G120D and A121D. Moreover, site-directed mutagenesis showed that double mutations G120R A121H and G120P A121P also increased resistance to penicillin and TC by 2-4 times [13].

The MtrC-MtrD-MtrE system was the first efflux system described in *N. gonorrhoeae*. It determines the resistance of gonococci to various antibacterial agents: penicillin, TC, macrolides, fluoroquinolones, and hydrophobic antibacterial agents produced by the host (fatty acids, antibacterial peptides) [9]. *MtrRCDE* locus of gonococcal chromosome contains a regulatory gene *mtrR* and 3 tandem genes *mtrC*, *mtrD* and *mtrE* encoding MtrC, MtrD and MtrE proteins, respectively. Transcription of MtrCDE complex proteins is regulated by MtrR repressor protein. Increased expression of MtrCDE complex can be determined by mutations in the promotor area of *mtrR* gene (deletion of adenine or insertion of two thymines in a 13 b.p. inverted repeat located between (-10) and (-35) positions of the promoter) or G45D substitution in the encoding region of *mtrR* gene [14].

The association between TC resistance and V57M mutation in *rpsJ* gene encoding S10 protein of 30S ribosomal subunit led to the formation of a mutant phenotype (MSC≥1μg/ml) [10]. The combination of this mutation with known mutations in MtrCDE locus and *penB* mutation produces a cumulative effect and increases resistance by 3-4 fold.

The aim of the present study was to evaluate the contribution of genetic mechanisms into the formation of phenotypic resistance of clinical *N. gonorrhoeae* strains to TC. For detection of mutations in *MtrRCDE* locus and *rpsJ* gene we used an approach based on minisequence reaction followed by identification of products by MALDI-TOF MS (matrix-assisted laser desorption time-of-flight mass spectrometry). The complex analysis of genetic markers was successfully used for the evaluation of *N. gonorrhoeae* resistance to fluoroquinolones and β-lactam antibiotics [1,3].

MATERIALS AND METHODS

Clinical strains of *N. gonorrhoeae* were isolated from patients with uncomplicated gonococcal infection from 3 regions of the Russian Federation: Central (Mocsow), Privolzhskii (Samara, Kazan'), and Siberian (Irkutsk). The isolated strains were confirmed to belong to *N. gonorrhoeae* species by biochemical methods using Crystal BBL system including 29 identification parameters.

MSC of TC was measured by the method of serial dilutions in agar using standard *N. gonorrhoeae* strain ATCC® 49226 as the control. The sensitivity of the isolated strains to the antibiotic was evaluated on the basis of CLSI criteria [5].

N. gonorrhoeae cells obtained after culturing on selective media were used for DNA isolation with DNA-express kits (Litekh Company).

Amplification of *por, mtrR, rpsJ* and *tet*(M) gene regions were carried out using primers listed in Table 1, for amplification of *mtrR* locus we used primers MtrPF and MtrPR chosen in such a way that PCR product contained the *mtrR* gene promoter sequence and a fragment of the gene.

Amplification was carried out in a reaction mixture containing 66 mM Tris-HCl (pH 9.0), 16.6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 100 μM each dNTP, 1 U Taq-polymerase (Promega), and 5 pmol each primer. The reaction was performed in DNA Engine Tetrad™ amplifier (MJ Research). The amplification profile for different target genes was universal and adapted for maximum yield of PCR product for all studied loci of the genome: 15 sec at 94°C, 15 sec at 58°C, and 15 sec at 72°C; 35 cycles.

The results of amplification of plasmid genes were analyzed in 2% agarose gel. The results demonstrate the presence or absence of plasmid resistance determinants in each strain.

For dephosphorylation of 5'-terminal phosphate groups of dNTP, the products of amplification of *mtrR* and *rpsJ* genes were incubated with 0.5 U arctic shrimp phosphatase (Promega) for 30 min at 37°C and then heated at 85°C for 10 min for inactivation of the enzyme.

Reaction of thermocyclic minisequencing was carried in a reaction mixture containing 66 mM Tris-HCl (pH 9.0), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM each dNTP and/or ddNTP, and 20 pmol each primer (Table. 2) and 2 U TermiPol DNA Polymerase (Solis Biodyne) using amplified *mtrR* and *rpsJ* gene fragments. The products of minisequencing were amplified by the following protocol: 20 sec at 94°C, 20 sec at 58°C, and 15 sec at 72°C; 70 cycles in a DNA Engine Tetrad™ (MJ Research) amplifier.

The products of minisequencing were purified using SpectroCLEAN Kit (Sequenom). An aliquot (0.2-1.0 μl) of purified sample was applied onto a dried AnchorChip target plate (400 μ; "Bruker Daltonics") prepared from saturated solution of 3-hydroxypicolinic acid (Fluka) in 50% acetonitrile (Merck) containing 10 g/liter dibasic ammonium citrate (Fluka) and dried on air. All solvents including water (Merck) were analytic pure grade or specially intended for mass spectrometry.

Mass spectrometry was carried out using a Microflex MALDI-TOF spectrometer (Bruker Dalto-

nics) equipped with a nitrogen laser (λ=337 nm) with pulse frequency of 20 Hz. All measurements were carried out in a linear regimen; positively changed ions were detected. For each mass spectrum recording we used 30 laser pulses with radiation power set at the level of minimum threshold value sufficient for desorption-ionization of the sample. Mass spectra were registered, processed, and analyzed using Bruker Daltonics software: flexControl 2.4 (Build 38) and flexAnalysis 2.4 (Build 11).

The presence of peaks corresponding to ions with the expected molecular weight reflected nucleotide context in the certain position (Table 2).

Nucleotide sequence of *por* gene was determined using a modified Sanger's method using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Hitachi). Full-length sequence of *por* gene was obtained by merging nucleotide fragments A and B using Vector NTI® Suite 9 No software (Informax Inc.).

Since we studied qualitative nominal signs (which cannot be arranged according to a certain semantic principle), the absence or presence of association between genotypes and sensitivity and the strength of relationships were evaluated using χ^2 test and Cramer's V test, respectively. The calculations were performed using Statistica 6.0 software. Statistic tests were performed at $p<0.05$ using the data of one-way analysis.

RESULTS

We used 129 clinical strains of *N. gonorrhoeae* from Central (32 strains), Privolzhskii (39 strains), and Siberian (58 strains) regions of the Russian Federation collected in 2005-2006. Microbiological analysis showed that 25 strains (19%) were TC-sensitive (MSC≤0.25), 65 strains (27%) exhibited medium sensitivity (MSC=0.5-1.0), and 39 (54%) were TC-resistant (MSC≥2), including 6 strains with MSC≥16 corresponding to TRNG phenotype.

The maximum number of TC-resistant *N. gonorrhoeae* strains were found in Central region (72%). In Privolzhskii and Siberian regions, resistant strains comprised 38 and 53%, respectively (Fig. 1, *a*).

Genetic analysis of known determinants of TC resistance was performed for all strains, in particular, the presence of substitutions in *mtrRCDE* locus leading to G45D mutation and/or delA(-35) and/or insTT(-10) denoted as $mtrR_{mut}$ genotype; substitutions in *rpsJ* gene leading to mutation V57M ($rpsJ_{mut}$ genotype); all substitution variants in *por* gene leading to mutations G120D, G120K, G120R and/or A121D, A121H, A121P ($penB_{mut}$ genotype); and the presence of *tet(M)* determinant ($tetM_{pres}$ genotype). $tetM_{pres}$ genotype was detected by PCR. $mtrR_{mut}$ and $rpsJ_{mut}$ genotypes were detected by minisequencing followed by measuring of the weight of the reaction products by mass spectrometry. The discriminating capacity of this approach is based on selective enzymatic elongation of oligonucleotide primers by 1, 2, or 3 nucleotide moieties depending on nucleotide sequence in the site of polymorphism followed by MALDI-TOF mass spectrometry (Table 2). $penB_{mut}$ genotype was detected by direct sequencing.

The incidence of detection of resistance markers among the analyzed strains were: *tetM* — 3.1%, mutations in genes *rpsJ* — 82.2%, *penB* — 62.8%,

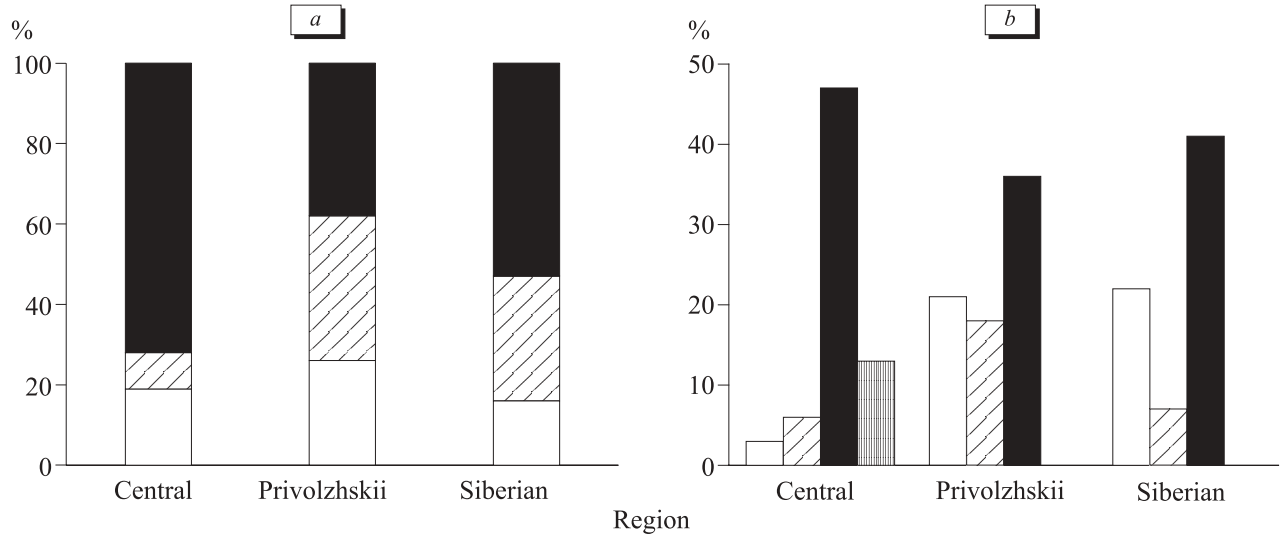

Fig. 1. Regional distribution of studied *N. gonorrhoeae* isolates. *a*) results of microbiological tests for TC sensitivity. Dark bars: resistant strains (MSC≥2 μg/ml); shaded bars: medium sensitivity (MSC=0.5-1.0 μg/ml); light bars: sensitive strains (MSCd"0.25 μg/ml). *b*) incidence of significant genetic markers of TC resistance. Light bars: wt, oblique shading: $rpsJ_{mut}$+$mtrR_{mut}$; dark bars: $rpsJ_{mut}$+$penB_{mut}$+$mtrR_{mut}$; vertical shading: $tetM_{pres}$.

TABLE 1. Primers Used for Amplification of *N. gonorrhoeae* Genome Fragments

Target	Primer, 5'-3' sequence	Product size, b.p.
por, fragment **A**	**M13-Por01**: GTCACGACGTTGTAAAACGACGGCCAGTCTGACTTTGGCAGCCCTT	
	M13R-Por08: CACACAGGAAACAGCTATGACCGTATTGTGCGAAGAAGC	500-600*
por, fragment **B**	**M13F-Por11**: GTCACGACGTTGTAAAACGACGGCCAGTCTGTCCGTACGCTACG	
	M13R-Por14: CACACAGGAAACAGCTATGACCAGATTAGAATTTGTGGGCGC	500-600*
rpsJ	**RPS-for**: GTGCTGTTGTAAAAGGCCCG	186
	RPS-rev: CGGCCGGCAAATCCAGCTTC	
mtrR	**MtrPF**: GCCAATCAACAGGCATTCTTA	401
	MtrPR: GTTGGAACAACGCGTCAAAC	
tet(M)	**TET-1new**: ATCCTTTCTGGGCTTCCATTG	436
	TET-2new: CCGAGCAGGGATTTCTCCAC	

Note. Sequences of M13F and M13R primers are underlined. *Lengths of amplified fragments vary for different serotypes.

and *mtrR* — 54.3%. In 17.5% cases the selected genetic marjers of TC resistance were absent. At the first stage, for evaluation of association between genotype (all analyzed genetic signs) and phenotype (resistance to antibiotic) we carried out statistical processing of the obtained data (Table 3) and revealed a significant correlation by Pearson's χ^2 test (63.3, $p<0.01$) and Cramer's V test (0.70).

Wild-type strains bearing no mutations in the analyzed loci had TC-sensitive phenotype in 73% cases and were characterized by medium sensitivity in 27% cases. This phenomenon can be explained by the existence of other molecular mechanisms of TC resistance, which were not analyzed in the present study. However, this significant ($p<0.01$) predominance of sensitive strains among wild-type genotype suggests that this sign (*wt* genotype) can be used as a marker of TC sensitivity.

Statistic analysis revealed no significant ($p<0.05$) differences in the incidence of TC sensitivity categories for strains with genotypes $mtrR_{mut}$, $rpsJ_{mut}$, or $rpsJ_{mut}$+$penB_{mut}$. Thus, the presence of $mtrR_{mut}$, $rpsJ_{mut}$, and $rpsJ_{mut}$+$penB_{mut}$ genotypes suggests that these signs cannot be used as markers of TC resistance. Our results did not confirm the data [10] that the presence of at least one marker $rpsJ_{mut}$ determines medium resistance of *N. gonorrhoeae* to TC at the level of 1 μg/ml.

The group of strains with genotypes $rpsJ_{mut}$+$mtrR_{mut}$ or $rpsJ_{mut}$+$penB_{mut}$+$mtrR_{mut}$ significantly

TABLE 2. Primers Used for Detection of Mutations in Genes *mtrR* and *rpsJ* and Expected Weights of Minisequencing Reaction Products

Gene	Nucleotide context[1]	Primer, 5'-3' sequence, molecular weight	Molecular weight of wild type product[2]	Mutation	Molecular weight of mutant product[3]
rpsJ	**wt** CCGCAC(**G**)TG	RPS_Z:	6292 Da	Val57Met	6910 Da
	mutCCGCAC**(**A)T**G**	ACATTTTCCGTTCTCCGCAC	(+**ddG**)	GTG-ATG	(+dA+dT+**ddG**)
		5979 Da			
mtrR	**wt** GGAT**(**A**)**AAAA**G**	MtrR1:	7676 Da	delA-35	7363 Da
	mutGGAT**( )**AAAA**G**	ACATACACGATTGCACGGAT	(+5dA+**ddG**)		(+4dA+**ddG**)
		6110 Da			
	wt GTTTTTT**( )**ATA	mtrR4:	7474 Da	insTT-10	8101 Da
	mutGTTTTTT**(**TT**)**ATA	TTGACGAGGGCGGATTAT	(+6dA+**ddG**)		(+8dA+**ddG**)
		5595 Da			
	wt CG(G)CGCGCT	mtrR6:	6770 Da	Gly45Asp	6463 Da
	mut G(**A**)CGCGCT	TGAAATGCCAATAGAGCGCG	(+dC+dC+**ddG**)	GGC-GAC	(+**ddT**)
		6175 Da			

Note. [1]Position of 3' terminal of the primer is underlined; position of mutation is shown in parentheses. [2]ddNTP terminating minisequencing reaction are shown by bold letters. [3]dNTP and ddNTP participating in the reaction are shown in parentheses.

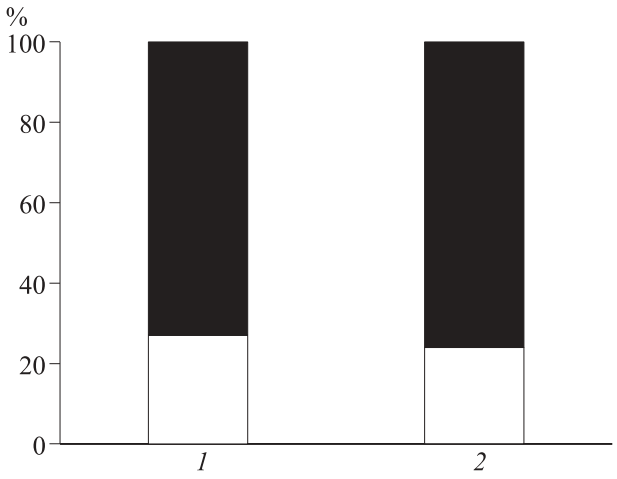

Fig. 2. Microbiological (*1*) and genetic (*2*) testing of TC sensitivity in the studied strains. Dark bars: resistant isolates; light bars: sensitive isolates.

($p<0.01$) characterizes the resistant genotype. Hence, detection of these genotypes can be used as the marker of TC resistance.

Combined genotype including any markers against the background of the presence of *tetM* determinant significantly ($p<0.01$) characterizes the resistant genotype. Thus, $tetM_{pres}$ genotype in combination with any other markers can be used for prediction of TC resistance with a probability approximating 100%.

Statistic analysis of combination of known genetic markers allowed us to select genotypes reliably predicting TC resistance ($p<0.01$): $rpsJ_{mut}$+$mtrR_{mut}$, $rpsJ_{mut}$+$penB_{mut}$+$mtrR_{mut}$, and $tetM_{pres}$.

Our conclusions on the possibility of using the analyzed genes as markers of TC resistance allowed us to refer the studied strains to the corresponding sensitivity category on the basis of their genotype. The obtained distribution into two sensitivity groups (Fig. 2) coincided ($p<0.01$) with the data of microbiological analysis.

Wild genotype was practically absent in Central region (1 strain), while in the Privolzhskii and Siberian regions it was relatively prevalent (8 and 13 strains, respectively, Fig 1, *b*). *N. gonorrhoeae* strains bearing *tetM* determinant plasmid gene were detected only in Central region (4 strains). No differences were revealed in regional distribution of other genotypes. On the whole, the resistance in Central region was higher than in Privolzhskii and Siberian regions. We revealed no correlations between *N. gonorrhoeae* resistance in different regions and the incidence of *tetM* determinant by statistical methods due to low incidence of this determinant. Therefore, the relatively high level of *N. gonorrhoeae* resistance to TC in Central region can be related to its economic and demographic peculiarities. It should be noted that chromosome mechanisms of resistance predominate in all regions, and only in Central region plasmid factors make a minor contribution into resistance formation.

Thus, the chosen genotypes and modern biomolecular mechanisms of complex evaluation of TC sensitivity in clinical gonococcus strains can be used for prediction of phenotypical resistance of *N. gonorrhoeae*.

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TABLE 3. Correlation of Genotypes (Combination of Genetic Markers of Resistance) and Categories of TC resistance in Analyzed *N. gonorrhoeae* Strains

Genotype	*n*	Phenotype (MSC, μg/ml)		
		<0.25	0.5-1.0	>2
Wild type (wt)	22	16 (73%)	6 (27%)	0*
Mutant genotypes				
$rpsJ_{mut}$+$mtrR_{mut}$	13	0	8 (62%)	5 (38%)*
$rpsJ_{mut}$+$penB_{mut}$	25	4 (16%)	9 (36%)	12 (48%)
$rpsJ_{mut}$+$penB_{mut}$+$mtrR_{mut}$	53	0	9 (17%)	44 (83%)*
$rpsJ_{mut}$	11	4 (36%)	3 (27%)	4 (36%)
$mtrR_{mut}$	1	0	0	0
$tetM_{pres}$ (combined genotype)	4	0	0	4* (100%)
Total	129	25 (27%)	35 (27%)	69 (54%)

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