Analysis of Genetic Markers of *N. Gonorrhoeae* Resistance to β-Lactam Antibiotics

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A complex method for detection of genetic markers of *N. gonorrhoeae* resistance to penicillin was developed. Mutations in *penA* and *ponA* genes were detected by minisequencing reaction with subsequent detection of reaction products by MALDI-TOF mass spectrometry. This approach was tested on 31 clinical strains of *N. gonorrhoeae* with minimum inhibitory concentration of penicillin from 0.03 to 8 μg/ml and higher. Mutations in *penA* and *ponA* genes in moderately resistant strains were shown (minimum inhibitory concentration up to 0.5 μg/ml) and mutations in *penA*, *ponA*, and *penB* genes in resistant strains (minimum inhibitory concentration more than 1.0 μg/ml). β-Lactamase genes were detected in 4 strains with high resistance (minimum inhibitory concentration 4-8 and more μg/ml). Correlation between microbiological resistance and presence of respective mutations in the studied locuses was detected.

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

Penicillin, antibiotic of choice in the treatment of gonococcal infections for a long time, is no longer recommended in the majority of countries because of great number of resistant strains. However, this drug is still used in many regions of Russia for the treatment of uncomplicated gonorrhea. The development of effective method for evaluation of resistance of *N. gonorrhoeae* strains, alternative to the microbiological method, can promote regional monitoring of the bacterium resistance and prescription of adequate therapy.

Penicillin is a β-lactam antibiotic inhibiting transpeptidase enzymes participating in the production of gram-negative bacterial cell wall peptidoglycan. These enzymes are sometimes called penicillin-binding proteins (PBP) because of their affinity for penicillin. Four PBP were found in *N. gonorrhoeae* cells, but only PBP 1 and PBP 2 are the main targets for the antibiotic [2].

Two groups of mechanisms of *N. gonorrhoeae* antibiotic resistance development are known, classified by the gene location into plasmid and chromosome mechanisms.

Plasmid resistance is caused by transfer (within mobile genetic elements) of *bla* gene encoding TEM-1 enzyme β-lactamase destroying the antibiotic β-lactam ring. Six types of plasmids containing TEM-1 β-lactamase gene and providing high resistance of *N. gonorrhoeae* to penicillin are known: Asia (4.2 MD), Africa (3.2 MD), Toronto (3.05 MD), Rio (2.9 MD), Nimes (3.8 MD), and New Zealand (6.5 MD) types [9,10].

Chromosome mechanisms of resistance development include mutation changes in PBP 1 and PBP 2 decreasing their affinity for penicillin. Insertion of aspartic acid codone in *penA* gene between amino acid positions 345 and 346 reduces PBP 2 affinity for penicillin and leads to the formation of intermediate resistance (minimum inhibitory con-

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centration (MIC) 0.120-0.125 μg/ml) [4]. Point mutation in *ponA* gene leading to substitution of Leu for Pro in amino acid position 421 reduces PBP 1 affinity for penicillin and, according to published data, causes high resistance (MIC 4 μg/ml) [11].

In addition to modification of target proteins, nonspecific mechanisms participate in the resistance formation: changes in the cell wall permeability and/or efflux. For penicillin it is modification of outer membrane proteins (porins) reducing antibiotic diffusion into the periplasmic space. Some mutations in *penB* locus encoding a site of loop 3 in Por IB protein cause *N. gonorrhoeae* resistance to penicillins and tetracyclines [6]. The most important of them are Gly120Lys and double replacement (Gly120Asp, Ala121Asp). The effects of Gly120Asp, Ala121Asp, or Ala121Asn mutations are less pronounced. Other variants are rare and are negligible for the formation of the gonococcus resistant phenotype [8].

The involvement of active efflux systems in the development of *N. gonorrhoeae* resistance to penicillin is not proven. Published data are contradictory: some authors claim that mutations in the *mtrR* gene and its promotor increase resistance to 0.2 μg/ml [12], others consider that penicillin MIC does not change [11].

We evaluated the possibility of predicting microbiological resistance of *N. gonorrhoeae* strains by means of analysis of genetic markers of β-lactam antibiotic resistance by modern methods.

MATERIALS AND METHODS

Clinical strains of *N. gonorrhoeae* isolated from patients with suspected gonococcal infection were verified by biochemical methods using Crystall BBL test system, including 29 identification parameters. ATCCT^R 49226 strain served for control.

Penicillin MIC was determined by serial dilutions in agar [5] using standard *N. gonorrhoeae* ATCCT^R 49226 strain for control. Antibiotic sensitivity of the strains was evaluated in accordance with NCCLS criteria [7].

N. gonorrhoeae cells obtained by culturing in selective nutrient media were used for isolation of DNA by the method of R. Boom *et al.* [3].

Amplification of *bla*, *penA*, and *ponA* gene areas was carried out (Table 1). A 4-primer system was used for amplification of *bla* gene; this system detects the main varieties of this gene present in β-lactamase gene carrying plasmids. The reaction was carried out in a mixture containing 10 mM Tris HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 250 μM each dNTP, 1 U Taq-pol (Promega) and 10 pmol corresponding primers. Amplification was carried out in a Tertsik programmed thermostat (DNK-Tekhnologiya; TU 9452-001-46482062-98). Amplification products were analyzed in 2% agarose gel.

Dephosphorylation of dNTP 5'-terminal phosphate groups in the reaction mixture after amplification was carried out during incubation with 0.5 U Shrimp Alkaline Phosphatase (Fermentas) for 20 min at 37°C with subsequent inactivation of the enzyme by 10-min heating at 85°C.

Thermocyclic minisequencing reaction was carried out in 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 dGTP, 0.2 mM dTTP, 0.2 mM ddCTP, 20 pmol each primer (Table 2), and 2 U TermiPol DNA Polymerase (Solis Biodyne). Amplified fragments of *penA* and *ponA* genes served as the matrix. Minisequencing products were reproduced in a programmed thermostat (20 sec at 94°C, 20 sec at 58°C, 15 sec at 72°C; 70 cycles).

Minisequencing products were purified by SpectroCLEAN Kit (Sequenom) in accordance with the instruction. The adsorbent from the kit (8 mg) was dissolved in 15 μl ultrapure water (Merck). The resultant suspension was put into tube with minisequencing reaction products. The content of the tube was thoroughly mixed and incubated at room temperature for 15 min. The adsorbent was precipitated by centrifugation (5 min, 1000 *rpm*). Supernatant was used for mass spectrometric analysis.

An aliquot of the sample (0.2-1.0 μl) with 10-30 pmol/μl oligonucleotides, obtained after purification, was applied onto the matrix dried on AnchorChip target (400 μ, Bruker Daltonics), prepared from saturated solution of 3-hydroxypicolinic acid (Fluka) in 50% acetonitrile (Merck) with 10 g/liter dibasic ammonium citrate (Fluka) and dried in the air. All solvents, including water (Merck), were analytically pure or special for mass-spectrometry.

The results of *penA* and *ponA* genes minisequencing by mass spectrometry were recorded on a Reflex IV device (Bruker Daltonics) in a linear mode using a nitrogen laser (λ=337 nm and pulse frequency 9 Hz in the positive ion mode). The analyzer delay time was 200 nsec, accelerating electrode voltage 20.0 kV, accumulating electrode voltage 17.1 kV, and focusing lens voltage 9.4 kV. The mass-spectrometer parameters were optimized for the mass/charge (m/z) range 1000-10,000. Calibration constants were determined using peptide mass spectra. Each oligonucleotide mass spectrum was obtained at 30 accumulations at constant power of laser at the level of threshold value for the resolution increase.

TABLE 1. Primers and Amplification Profiles for Various Target Genes

Target	Primer	Product size	Amplification profile
bla	B1-f - 5'-TACTCAATCGGTAATTGGCT-3' D2-r - 5'-GCCCAAAAAAGGGACGAAAG-3'	B3-f - 5'-CGTATATCTAGTTGAGGCAC-3' D4-r - 5'-GTGCCTCAACTAGATATACG-3'	340 n. p. (Asia, Africa) 142 n. p. (Toronto) 94°C — 20 sec, 58°C — 10 sec, 72°C — 10 sec; 35 cycles
penA	penA-f: 5'-CGTGATTGCGAAGGCATTGG-3'	penA-r: 5'-GTGCGTCAGTGCGGTATAGG-3'	379 n. p. 94°C — 10 sec, 65°C — 10 sec, 72°C — 10 sec; 35 cycles
ponA	PonA1-f: 5'-GAGAAAATGGGGGAGGACCG-3'	PonA1-r: 5'-GGCTGCCGCATTGCCTGAAC-3'	206 n. p.

The genotype of the studied sample was determined as mutant or wild from the presence of ions of certain molecular weight in reaction product mass spectra (Table 2).

Nucleotide sequences of *por* gene in the studied strains were determined previously [1]. Amino acid sequences of Por protein were derived from the known nucleotide sequences using Vector NTIT^R Suite v.6.0 software (Informax Inc.).

RESULTS

Thirty-one clinical strains of *N. gonorrhoeae* with penicillin MIC from 0.03 to 8 μg/ml and higher were selected from laboratory bank of strains. Primers for detection of three most prevalent types (Asia, Africa, and Toronto) were selected by the results of comparative analysis of nucleotide sequences of β-lactamase TEM-1 gene containing plasmids present in Gene Bank. β-Lactamase genes were detected in 4 strains with high resistance (MIC 4-8 and more μg/ml) by the results of amplification.

Two oligonucleotide primers were selected with consideration for the data of comparative analysis of nucleotide sequences of amplified fragments of *penA* and *ponA* genes (Table 2); binding of several nucleotides to 3' terminals of these primers allowed detection of the corresponding mutations in the studied genes. Estimated weights of minisequencing reaction products for each genotype variant are presented in Table 2. Minisequencing reaction with subsequent evaluation of the reaction product weights was used for registration of nucleotide replacements in *penA* and *ponA* genes. The discriminating capacity of the method is based on selective enzymatic elongation of oligonucleotide primers by 1, 2, or 3 nucleotide components, depending on the nucleotide sequence at the site of polymorphism. Analytical capacity of Reflex IV MALDI-TOF mass-spectrometer (Bruker Daltonics) effectively discriminated oligonucleotides 10-30 nucleotides long differing by at least one nucleotide component (about 300 Da), which is in complete agreement with our task.

Mass spectra of the reaction products in the presence of mutations in *penA* and *ponA* and wild type genes are presented in Fig. 1. Primers for the analysis of *penA* and *ponA* genotypes were selected so that minisequencing reaction products differed from each other. This method was used for the analysis of genetic polymorphism of a sampling of *N. gonorrhoeae* clinical strains with a known MIC for penicillin (Table 3).

Analysis of Por IB protein amino acid sequence revealed the appearance of mutations in positions 120 and 121 of the studied strains with increasing penicillin MIC (Table 3). These mutations were associated with decreased cell membrane permeability and development of resistance to antibacterial drugs [6,8].

The data for all analyzed locuses are presented (Table 3). All penicillin-sensitive strains (MIC≤0.06 μg/ml) had no mutations in any of the locuses. Mutation in *penA* was detected in moderately resistant strains, which was in line with published data [11].

TABLE 2. Expected Weights of *penA* and *ponA* Genes Minisequencing Reaction Products

Gene	Primer	Primer weight	Product weight, wild type	Product weight, mutant
penA	penAz 3'-GGGGTAAACATGGGTATCG-5'	5933 Da	6206 Da (+ddC)	6511 Da (+dT+ddC)
ponA	ponAz 5'-GGTTCAAGAGCCGTTGC-3'-	5227 Da	6133 Da (+dT+dG+ddC)	5500 Da (+ddC)

TABLE 3. Summary Analysis of Mutations in PBP 1, PBP 2, and Por Proteins and Presence of β-Lactamase Gene in the Studied *N. Gonorrhoeae* Strains

Strain	Penicillin MIC, μg/ml	PBP 2 (*penA*)	PBP 1 (*ponA*)	Por (*penB*)	β-Lactamase (*bla*)	Characterization
37z	0.03	Wild type	Wild type	Wild type		Sensitive
33p	0.03	Wild type	Wild type	Wild type		
55p	0.06	Wild type	Wild type	Wild type		
60p	0.06	Wild type	Wild type	Wild type		
35z	0.12	Asp-345a	Wild type	Wild type		Moderately resistant
39	0.25	Asp-345a	Wild type	Wild type		
26	0.25	Asp-345a	Leu421Pro	Wild type		
30	0.25	Asp-345a	Leu421Pro	Wild type		
34	0.25	Asp-345a	Leu421Pro	Wild type		
45	**0.25**	**Asp-345a**	**Leu421Pro**	**Gly120Lys;Ala121Asp**		
73	0.25	Asp-345a	Leu421Pro	Wild type		
95	0.25	Asp-345a	Leu421Pro	Wild type		
57	0.5	Asp-345a	Leu421Pro	IA серотип		
6z	**0.5**	**Asp-345a**	**Leu421Pro**	**Wild type**		
27z	1.0	Asp-345a	Leu421Pro	Ala121Asp		Resistant
41	1.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp		
70	**1.0**	**Asp-345a**	**Wild type**	**Gly120Asp**		
79	1.0	Asp-345a	Leu421Pro	Gly120Asp		
89	1.0	Asp-345a	Leu421Pro	Gly120Asn;Ala121Asp		
94	**1.0**	**Asp-345a**	**Leu421Pro**	**Wild type**		
96	1.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp		
29c	2.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asn		
69	2.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp		
8z	4.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asn		
18c	4.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp		
38p	4.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp	+	
40p	4.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asn		
17z	8.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asn		
41p	8.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp	+	
49p	>8.0	Asp-345a	Leu421Pro	Gly120Lys;Ala121Asp	+	
42p	>8.0	Asp-345a	Leu421Pro	IA serotype	+	

According to our findings, mutation in *ponA* does not provide so high resistance as was reported previously [11]. Combined mutations in *penA* and *ponA* locuses were detected in moderately resistant strains with MIC≤0.5 μg/ml. Resistant strains (MIC>1.0 μg/ml) had significant mutations in all three studied locuses (*penA*, *ponA*, and *penB*). However, one of the 10 studied strains (strain "45") with MIC≤0.5 μg/ml had mutations in *penA*, *ponA*, and *penB*, but did not exhibit high resistance characteristic of this genotype. Presumably, this strain had changes in the genome, leveling the effect of the detected mutation in *penB* on the rate of penicillin diffusion into the cell. Strain "94" without mutations in *penB* locus exhibited high resistance (MIC 1.0 μg/ml). Presumably, this strain realizes other mechanisms of resistance formation, not analyzed in our study.

Hence, we showed the possibility of detecting genetic markers of *N. gonorrhoeae* resistance to penicillins by the minisequencing reaction and MALDI-TOF mass-spectrometric analysis for registration of the results. The relationship between microbiological resistance and presence of certain genetic mutations in the studied locuses was shown

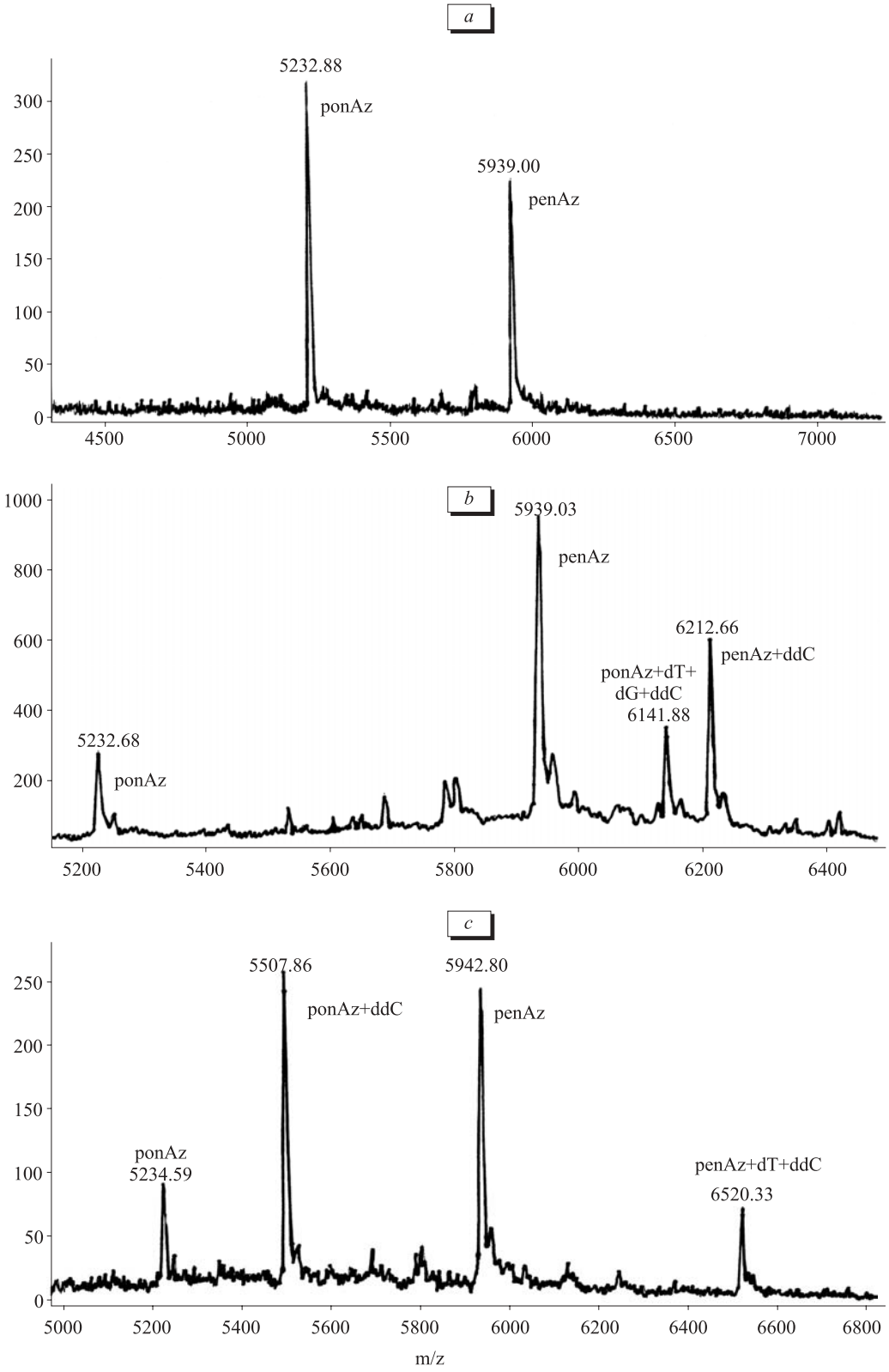

Fig. 1. Mass spectra of *penA* and *ponA* genes minisequencing reaction products. *a*) initial oligonucleotide primers; *b*) products of primer completion after detection of wild genotypes by *penA* and *ponA*; *c*) products of primer completion after detection of mutant genotypes by *penA* and *ponA*.

for some clinical strains of *N. gonorrhoeae*, this indicating prognostic significance of the analyzed genetic determinants.

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