GENETICS

Direct Evaluation of Drug Resistance Parameters in Gonococcus

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We carried out complex genetic analysis of clinical samples containing *N. gonorrhoeae* DNA, the genotype and profile of drug resistance of this agent were evaluated. Changes in genes responsible for the formation of *N. gonorrhoeae* resistance to penicillins, fluoroquinolones, and spectinomycin were detected during minisequencing with subsequent MALDI-TOF mass spectrometry. The sensitivity of gonococcus was evaluated directly in the clinical sample without culturing.

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

The problem of antibiotic resistance of *N. gonorrhoeae* impedes effective control of gonococcal infection. The best approach is the choice of drugs for the treatment of gonorrhea based on the data on individual sensitivity of *N. gonorrhoeae* strain. However, this approach is difficult to realize practically. Available methods for evaluation of the gonococcus sensitivity to antibiotics are very difficult and expensive, and therefore cannot become routine tests in the diagnosis of gonorrhea. This was demonstrated by the experience gained in Western Europe and the USA.

Molecular methods for the analysis of the agent requiring no culturing of gonococcus in nutrient media and maintenance of the microorganism viability until the analysis and permitting automated realization of the diagnostic process appeared in the early 1990s and were actively developed. The overwhelming majority of these methods involve detection of genetic markers of certain adaptive changes in the bacteria forming the mechanism of its antibiotic resistance.

The formation of the gonococcus resistance to antibacterial drugs is associated with the presence of groups of genes or changes in these genes. For example, penicillin (PC) resistance is caused by transfer of *bla* gene encoding TEM-1 β-lactamase enzyme with mobile genetic elements [10]. Mutations in *penA* and *ponA* genes [4,12] encoding PC-binding proteins 2 and 1, respectively, and changed permeability of cell wall because of mutations in the *penB* locus encoding a site in porin protein loop 3 [7] also lead to the resistance phenomenon.

Gonococcus resistance to quinolones develops because of mutations in *gyrA* and *parC* genes leading to amino acid substitutions in the corresponding subunits of DNA gyrase and topoisomerase [5,14]. No mutations in *gyrB* gene were detected, while mutations in *parE* gene, according to published data, do not correlate with increase of the level of drug resistance [8]. Enhanced activity of the efflux system in bacterial cell because of single nucleotide mutations in the promotor regions of *mtrR* [11] and *norM*

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genes [13] additionally increases quinolone resistance.

A correlation between PC and fluoroquinolone (FQ) resistance of *N. gonorrhoeae* strains and genetic changes in the corresponding locuses of the bacterial genome was previously demonstrated [1,3].

Here we used methods of molecular analysis for gonococcus detection and evaluation of its antibiotic sensitivity directly in the clinical material without preliminary culturing.

MATERIALS AND METHODS

The study was carried out on clinical samples from patients with suspected sexually-transmitted infections (cervical canal or urethral scrapings). Total DNA was isolated using DNA-express commercial kit (Litech Firm). *N. gonorrhoeae* DNA was detected in the studied material by PCR with Gonopol kit (Litech Firm).

Penicillin resistance of *N. gonorrhoeae* was evaluated by genetic analysis of *bla*, *por*, *ponA*, and *penA* genes [3]. Gonococcus genotype was determined by evaluating the *por* gene nucleotide sequence [2].

Genetic analysis of *N. gonorrhoeae gyrA*, *parC*, *norM*, *mtrR* genes associated with FQ resistance was carried out by PCR method followed by minisequencing and mass-spectrometry [1].

The *rrs* gene (16S RNA) was analyzed similarly; C1192->T substitution in this gene is responsible for gonococcus resistance to spectinomycin [6]. PCR was carried out with 16Sfor (5' aaggccgtt gccaatatcg 3') and 16Srev (5' tgtatgacgtgtgaagccc 3') primers. The resultant fragment (785 b. p.) was analyzed by minisequencing with 16SZ probe (5' ggc catgaggacttgac 3') and mass-spectrometric detection.

All amplification procedures were carried out in a DNA Engine TETRAD 2 programmed thermostat (MJ Research). The amplification products were analyzed in 2% agarose gel, if necessary.

The results of minisequencing of *ponA*, *penA*, *gyrA*, *parC*, *norM*, *mtrR*, and *rrs* genes were registered by MALDI-TOF mass-spectrometry in the linear mode on Reflex IV device (Bruker Daltonics), using nitrogen laser at λ=337 nm and pulse frequency 9 Hz in the positive ion mode.

The mutant or wild genotype of the studied sample was identified by the presence of ions of certain molecular weight in mass spectra of the minisequencing products.

RESULTS

A total of 12 samples containing *N. gonorrhoeae* DNA (according to PCR analysis) were selected of samples analyzed at clinical diagnostic laboratory of Institute of Dermatovenerological Diseases.

Amplification of 10 genetic locuses of *N. gonorrhoeae* was carried out with total DNA isolated directly from biological material as the matrix. Amplification results detected *N. gonorrhoeae bla* genes (components of Asia or Africa plasmids) in 2 strains.

Genetic analysis of *por* gene identified the Por type of *N. gonorrhoeae* and detected characteristic genetic mutations in *penB* locus (mutations in the Por protein positions 120 and 121) associated decreased membrane permeability and leading to PC resistance [9].

According to the results of Por typing, all the studied gonococcal DNA specimens were referred to the PIB serovar: PIB1 genotype was detected in 1 sample, PIB2 in 7, PIB3 in 3, and PIB22 in 1 sample. The results of genotyping indicate homogeneity of the studied *N. gonorrhoeae* population within each clinical sample, which appreciably increased the significance of information about the detected genetic markers of resistance.

Detection of oligonucleotide polymorphisms in *penA*, *ponA*, *gyrA*, *parC*, *norM*, *mtrR* or *rss* genes associated with PC, FQ, or spectinomycin resistance was carried out (Table 1).

Of the analyzed DNA samples, Nos. 1 and 2 had wild type by virtually all locuses, which gives us grounds to assume that the bacterium is sensitive to β-lactam antibiotics, FQ, and spectinomycin. Mutation in the promotor region of *mtrR* gene in sample No. 2 indicates initiation of nonspecific efflux processes, which can lead to moderate tetracycline resistance.

The absence of the *rrs* (16S) mutant genotype in the analyzed sampling attests to sensitivity to spectinomycin in all cases. Another picture was seen in the analysis of gene cassettes responsible for the formation of PC and FQ resistance (Table 1). Ten of the studied 12 DNA samples had this or that mutant genotype determining the drug resistance phenomenon. Moderate drug resistance to β-lactams with minimum suppressing concentration (MSC) of PC $\leq$0.5 μg/ml should be expected in 3 cases (Nos. 6-8 with genotype *bla*$^{-}$, *ponA*^{mut}, *penA*^{mut}, *penB*^{wt}). The expected MSC of PC for samples Nos. 3, 4, 10-12 with genotype *bla*$^{-}$, *ponA*^{mut}, *penA*^{mut}, *penB*^{mut} is 1-2 μg/ml and is characteristic of *N. gonorrhoeae* realizing the chromosome mechanisms of PC resistance. Gonococcal β-lactamase TEM-1 gene was found in specimens Nos. 5 and 9, which attests to high resistance (MSC>4 μg/ml) of the detected agent to β-lactam antibiotics. The detection of *bla* plasmid gene in specimen No. 9, along with the presence of mutations in *ponA* and

TABLE 1. Results of Genetic Analysis of *N. gonorrhoeae* DNA (Genotypes for Each Marker Locus)

DNA sample No.	*Por* type	Gene cassette responsible for PC resistance formation					Gene cassette responsible for FQ resistance formation				Gene responsible for spectinomycin resistance formation
		bla	*ponA*	*penA*	*penB*	*gyrA*	*parC*	*norM* promotor	*mtrR*	*mtrR* promotor	*rrs (16S)*
1	IB3	—	wt	wt	wt	wt	wt	wt	wt	wt	wt
2	IB2	—	wt	wt	wt	wt	wt	wt	wt	delA	wt
3	IB22	—	L421P	D-345a	G120K A121D	wt	wt	wt	wt	wt	wt
4	IB2	—	L421P	D-345a	G120KA 121D	wt	wt	wt	wt	delA	wt
5	IB2	+	L421P	D-345a	G120K A121N	wt	wt	wt	wt	wt	wt
6	IB3	—	L421P	D-345a	wt	D95G, S91F	wt	wt	wt	wt	wt
7	IB2	—	L421P	D-345a	wt	D95G, S91F	wt	wt	G45D	delA	wt
8	IB3	—	L421P	D-345a	wt	D95G, S91F	S87R	wt	wt	wt	wt
9	IB1	+	L421P	D-345a	wt	D95G, S91F	wt	wt	wt	delA	wt
10	IB2	—	L421P	D-345a	G120K A121N	D95G, S91F	wt	wt	wt	delA	wt
11	IB2	—	L421P	D-345a	G120K A121N	D95G, S91F	wt	wt	wt	delA	wt
12	IB2	—	L421P	D-345a	G120K A121D	D95G, S91F	wt	wt	wt	delA	wt

penA genes indicates the independence of plasmid and chromosome mechanisms of PC resistance formation.

Analysis of genetic markers of FQ resistance helped us to predict sensitive phenotype of *N. gonorrhoeae* in specimens Nos. 1-5. FQ sensitivity in specimens Nos. 3-5 is associated wit sharply pronounced PC resistance. Other gonococcal DNA specimens exhibited combined mutations in the *gyrA* gene corresponding to changes in amino acid sequence Ser91Phe and Asp95-Gly characteristic of strains with high FQ resistance (MSC>4 μg/ml). The detection of an additional mutation in the gonococcal *parC* gene in sample No. 8 suggests higher MSC for FQ: ≥8 μg/ml. The same is true for sample No. 7 with mutant genotype by *mtrR* gene and its promotor region.

Hence, genome DNA of the bacterial agent can be analyzed directly in clinical material without heretofore obligatory stage of bacterium culturing. This approach opens new vistas for the analysis of human and animal pathogens that cannot be cultured and for introduction of new methods of gene diagnosis for indication and phenotypical characterization of the agent into practical medicine.

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