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Microarray technologies for analysis of genetic determinants of *Neisseria gonorrhoeae* antimicrobial resistance

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Background. The etiological agent of gonococcal infection is capable of rapid development of antimicrobial resistance. The proportion of drug-resistant *N. gonorrhoeae* isolates in the world is growing each year, which increases the likelihood of an incurable infection.
Purpose of the study. Updating data on the distribution of genetic determinants of resistance in isolates of the modern Russian population of *N. gonorrhoeae* to a number of antimicrobial drugs using hydrogel biochip technology.
Methods. The study included 360 *N. gonorrhoeae* isolates submitted to the State Research Center for Dermatoveneorology and Cosmetology of the Ministry of Health of Russia from specialized dermatoveneorological medical organizations of eight constituent entities of the Russian Federation in 2019–2023. The susceptibility of *N. gonorrhoeae* to penicillin, ceftriaxone, tetracycline, azithromycin, and ciprofloxacin was determined by serial dilution in agar, with estimation of the minimum inhibitory concentration (MIC). The genetic determinants of *N. gonorrhoeae* resistance to antimicrobial drugs were identified using hydrogel biochip technology.
Results. The current data on the distribution of genetic determinants of *N. gonorrhoea* resistance to antimicrobial drugs are presented. In the Russian population of *N. gonorrhoea*, active processes are occurring that result in redistribution of the proportions of isolates that are resistant to different antimicrobial drugs. Since 2020, azithromycin resistance has increased, as well as there has been an increase in the proportion of isolates resistant to ciprofloxacin, sensitivity to penicillin has been restored, while the whole population remains sensitive to ceftriaxone. The validated biochip-based “NG-TEST” reagent kit provides a rapid determination of the *N. gonorrhoeae* resistance to ceftriaxone through the simultaneous identification of genetic determinants in the *penA*, *ponA*, and *porB* genes, as well as the calculation of the MIC value.
Conclusions. Microarray technologies for the identification of genetic determinants of *N. gonorrhoeae* antimicrobial resistance can be used as an auxiliary tool for the identification of resistant strains. The results of microarray-based analysis contribute to the selection of a treatment strategy for patients and provide an opportunity to monitor the molecular-epidemiological picture at the population level.

Keywords: ***Neisseria gonorrhoeae*; antimicrobial resistance; oligonucleotide microarrays**
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Микрочиповые технологии для анализа генетических детерминант устойчивости *Neisseria gonorrhoeae* к противомикробным препаратам

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Обоснование. Возбудитель гонококковой инфекции способен быстро формировать устойчивость к противомикробным препаратам. Доля лекарственно-устойчивых изолятов *Neisseria gonorrhoeae* в мире растет с каждым годом, что повышает вероятность возникновения неизлечимой инфекции.
Цель исследования. Актуализация данных о распространении генетических детерминант устойчивости изолятов современной российской популяции *N. gonorrhoeae* к ряду противомикробных препаратов с использованием технологии гидрогелевых биочипов.
Методы. В исследование включено 360 изолятов *N. gonorrhoeae*, поступивших с 2019 по 2023 г. в ФГБУ «ГНЦДК» Минздрава России из специализированных медицинских организаций дерматовенерологического профиля восьми субъектов Российской Федерации. Тестирование чувствительности *N. gonorrhoeae* к пенициллину, цефтриаксону, тетрациклину, азитромицину и ципрофлоксацину осуществляли методом серийных разведений в агаре с определением минимальных подавляющих концентраций (МПК). Идентификацию генетических детерминант устойчивости *N. gonorrhoeae* к противомикробным препаратам проводили с помощью технологии гидрогелевых биочипов.
Результаты. Представлены актуальные данные по распространению генетических детерминант устойчивости *N. gonorrhoeae* к противомикробным препаратам. В российской популяции *N. gonorrhoeae* происходят активные процессы, связанные с перераспределением долей изолятов, устойчивых к разным противомикробным препаратам. С 2020 г. возросла устойчивость к азитромицину, повышена доля изолятов, устойчивых к ципрофлоксацину, произошло восстановление чувствительности к пенициллину, при этом вся популяция остается чувствительной к цефтриаксону. Валидированный набор реагентов «NG-ТЕСТ» на основе биочипа обеспечивает быстрое определение устойчивости *N. gonorrhoeae* к цефтриаксону посредством одновременной идентификации генетических детерминант в генах *penA*, *ponA* и *porB* и расчета значения МПК.
Заключение. Микрочиповые технологии для идентификации детерминант устойчивости *N. gonorrhoeae* к противомикробным препаратам могут быть использованы в качестве вспомогательного инструмента выявления резистентных штаммов. Результаты анализа на биочипах способствуют выбору стратегии лечения пациентов и обеспечивают возможность наблюдения за молекулярно-эпидемиологической картиной на уровне популяций.

Ключевые слова: ***Neisseria gonorrhoeae*; устойчивость к противомикробным препаратам; олигонуклеотидные микрочипы**
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Background

Despite almost a hundred-year-long history of treatment of gonococcal infection with a variety of antimicrobial drugs, more than 80 million cases of this disease are recorded annually in the world [1]. The etiological agent of gonorrhea is the gram-negative bacterium *Neisseria gonorrhoeae*, which has an exceptional ability to develop antibiotic resistance [2]. The proportion of drug-resistant *N. gonorrhoeae strains* in the world increases every year; cases of unsuccessful treatment of gonococcal infection with recommended products due to super-resistant gonococcal variants are published regularly [2–4]. Epidemiological data show that the situation with gonococcal infection in Russia over two decades of the 21st century has been and remains better than in European countries and the USA, despite the general recent increase in incidence: in 2021 — an increase by 10.4 % compared to the previous year; in 2022 — by 10.0 %; during the first four months of 2023 — by 5.5 % compared to the same period in 2022 [5].

The product of choice for the treatment of gonococcal infection in the Russian Federation is ceftriaxone, a third generation cephalosporin. In the Russian gonococcal population, the proportion of ceftriaxone-resistant isolates is minimal; studies have reported detection of sensitive isolates only [6]. This condition is atypical against the background of a global trend towards an increase in detected cases of isolates resistant to third-generation cephalosporins [7–11]. Quite a difficult situation is observed in China, where the proportion of ceftriaxone-resistant isolates increased from 2.9 % in 2017 to 8.1 % in 2022 [7].

An analysis of the Russian *N. gonorrhoeae* population sensitivity to azithromycin (used as a component of combination treatment of gonococcal infection in a large number of countries, but not recommended for therapy in Russia) showed an increase in resistant isolates from 0 % in 2018–2019 to 17 % in 2020 and 9 % in 2021 [12]. This proportion of azithromycin-resistant isolates precludes its use in accordance with the World Health Organization (WHO) criterion, in which the total proportion of gonococcal strains sensitive to an antimicrobial drug should not be lower than 95 % [13]. At the same time, the obtained data on the *N. gonorrhoeae resistance* to azithromycin emphasize the need for further epidemiological surveillance for spread of new variants of the etiological agent of gonococcal infection in Russia.

In the current situation, studies aimed at annual monitoring of the Russian population of the etiological agent of gonococcal infection are quite relevant, including those aimed at identification of genetic determinants of resistance to both ceftriaxone and azithromycin, as well as to products previously used to treat gonorrhea — penicillin, tetracycline and ciprofloxacin. To solve the set tasks, the Institute of Molecular Biology of the Russian Academy of Sciences (IMB RAS), together with the State Research Center for Dermatovenereology and Cosmetology of the Ministry of Health of Russia (FSBI "SRCDC" of the Ministry of Health of Russia) have developed several generations of oligonucleotide hydrogel microarrays (biochips), providing accurate identification of the genetic determinants of *N. gonorrhoeae* resistance to various antimicrobial drugs [12, 14, 15]. Particular attention is paid to the analysis of ceftriaxone resistance, where, based on the results of identifying determinants using a biochip, a machine learning method is used to predict the minimum inhibitory concentration (MIC) of ceftriaxone [16]. The developed approach with the modifications described in this paper (i.e., molecular probes helping to determine belonging to the species *N. gonorrhoeae*) became the basis for the "NG-TEST" reagent kit for identification of the genetic determinants of resistance to the third-generation cephalosporins for the etiological agent of gonococcal infection *N. gonorrhoeae*.

The purpose of this study is to update data on the distribution of genetic determinants of resistance to a number of antimicrobial drugs for the modern (2019–2023) Russian population of *N. gonorrhoeae* isolates using hydrogel biochip technology.

Methods

Study objects

The study included 360 clinical isolates of *N. gonorrhoeae* submitted to the reference center of the FSBI "SRCDC" of the Ministry of Health of Russia as part of surveillance for antimicrobial resistance of sexually transmitted infections, including 123 clinical isolates in 2019; 119 — in 2020; 52 — in 2021; 25 — in 2022; 42 — in 2023. The samples were received from specialized dermatovenereological medical institutions located in eight constituent entities of the Russian Federation, belonging to five federal districts: Central — Moscow (n = 17) and Kaluga region (n = 101); Northwestern — Arkhangelsk region (n = 29); Southern — Astrakhan region (n = 23); Volga Federal District — the Republic of Tatarstan (n = 20) and the Chuvash Republic (n = 76); Siberian — Omsk (n = 20) and Novosibirsk (n = 74) regions.

Primary identification of *N. gonorrhoeae* isolates was carried out in the region of their isolation based on the results of microscopic examination and oxidase test [17–19]. The final verification was carried out at the reference center of the FSBI «SRCDC» of the Ministry of Health of Russia using NH cards on a VITEK 2 Compact analyzer (bioMérieux, France). For gram-negative oxidase-positive diplococci assessed as *N. gonorrhoeae* with a probability of less than 99 % based on a combination of biochemical properties, a supportive confirmatory test was carried out using a MALDI Microflex time-of-flight mass spectrometer with ionization (Bruker Daltonics GmbH, Germany).

Antimicrobial sensitivity testing

Determination of *N. gonorrhoeae* strains sensitivity to antimicrobial drugs was carried out using the method of serial dilutions in agar in accordance with the standard procedure [20] using the control strain *N. gonorrhoeae* ATCC 49 226 from the collection of type cultures of microorganisms. The assessment of *N. gonorrhoeae* sensitivity to antimicrobial drugs was performed in accordance with the criteria of MUK (methodology regulations) 4.2.1890-04 (https://fcgie.ru/download/elektronnaya_baza_metod_dokum/muk_1890-04.pdf, accessed on September 12, 2024) for all antimicrobial drugs, except azithromycin, for which the EUCAST criteria were applied (The European Committee on Antimicrobial Susceptibility Testing, Version 14.0, http://www.eucast.org, accessed on September 12, 2024). Antimicrobial sensitivity criteria for *N. gonorrhoeae* are presented in Table 1.

Identification of genetic determinants of N. gonorrhoeae antimicrobial resistance

Bacterial DNA was isolated using the "DNA-Express" kit (Litech, Russia), DNA concentration was assessed using a Qubit 3.0 spectrofluorometer (Invitrogen, USA).

Table 1. Criteria of phenotypic sensitivity of *N. gonorrhoeae* to antimicrobial drugs
Таблица 1. Критерии фенотипической чувствительности *N. gonorrhoeae* к противомикробным препаратам

Antimicrobial drug (S; MR; R, mg/L)
Penicillin (≤ 0.06; 0.12–1.0; ≥ 2.0)
Ceftriaxone (≤ 0.25; – ; > 0.25)
Tetracycline (≤ 0.25; 0.5–1.0; ≥ 2.0)
Azithromycin (≤ 1.0; – ; > 1.0)
Ciprofloxacin (≤ 0.03; 0.06; > 0.06)

Note. S — sensitive; MR — moderately resistant; R — resistant. Azithromycin is always used in combination with another effective agent. MUK 4.2.1890-04 does not contain any indications for azithromycin, therefore EUCAST 14.0 was used, in which the ECOFF resistance threshold is 1 mg/l.

Примечание. Ч — чувствительный; УР — умеренно резистентный; Р — резистентный. Азитромицин всегда используется в сочетании с другим эффективным средством (цефтриаксоном или цефиксимом). В МУК 4.2.1890-04 отсутствуют указания об азитромицине, в связи с чем использовали EUCAST 14.0, в котором порог устойчивости ECOFF составляет 1 мг/л.

Analysis of genetic determinants associated with ceftriaxone resistance was carried out using production samples of the NG-TEST reagent kit. A specialized hydrogel biochip (Fig. 1) provided simultaneous identification of the following determinants:

- insertion of an aspartic acid codon at position 345–346 of the *penA* gene and Ala311Val; Ile312Met; Val316Thr, Pro; Thr483Ser; Ala501Val, Thr, Pro; Asn512Tyr; Gly542Ser; Gly545Ser, and Pro551Leu, Ser substitutions in mosaic and non-mosaic alleles of the *penA* gene;
- Leu421Pro substitution in the *ponA* gene;
- Gly120Lys, Arg, Asp, Asn, Thr, and Ala121Asp, Asn, Gly, Val, Ser substitutions in the *porB* gene.

The biochip also included molecular probes to detect *N. gonorrhoeae* species-specific polymorphisms in the *ISNgo2* transposable element (see Fig. 1).

Elements of the biochip are represented in the form of circles, highlighted in different colors based on the locus analyzed, and the detectable marker is indicated inside them. Elements with probe sequences corresponding to the wild type are highlighted with a thick line. Elements with

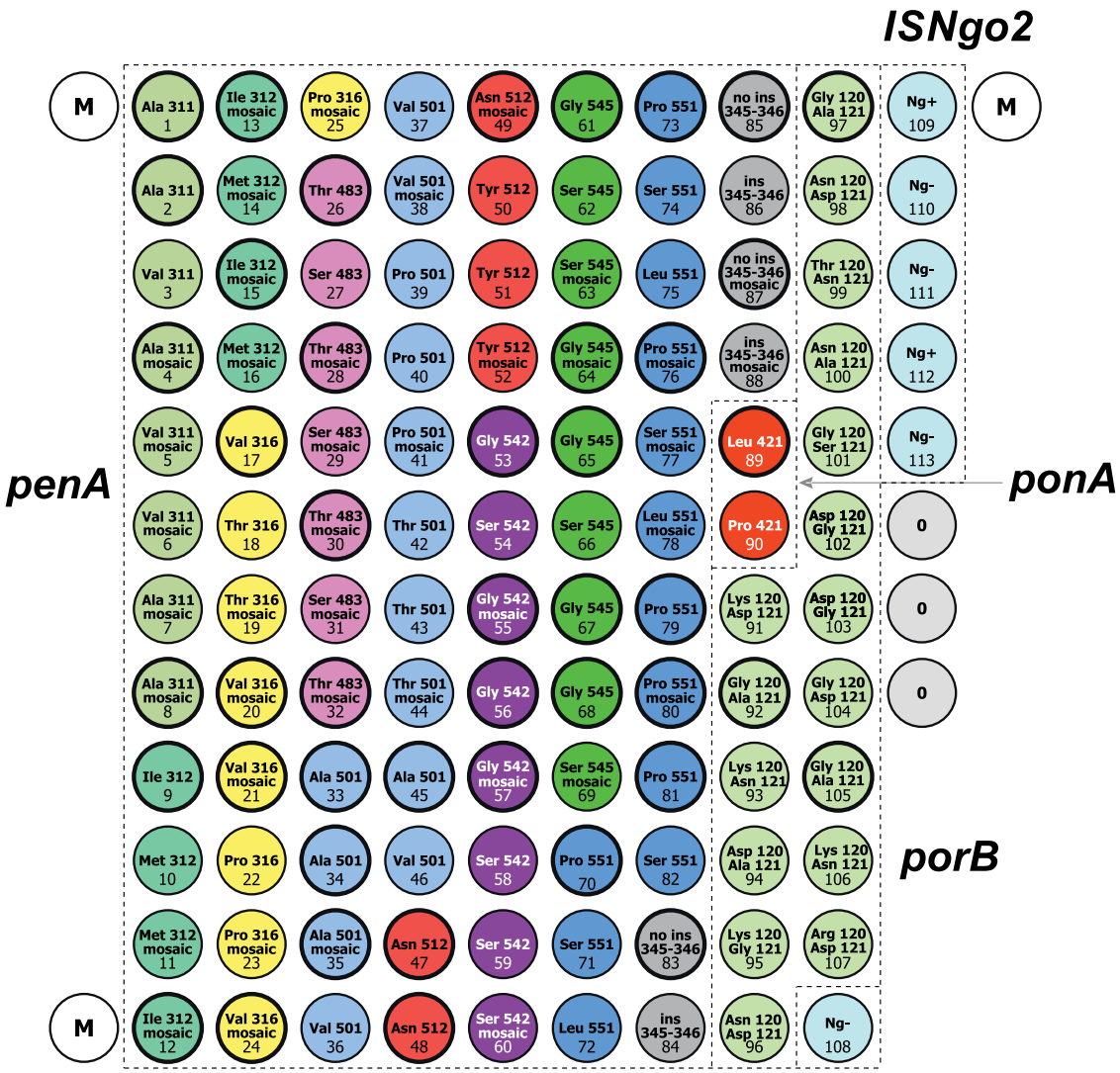


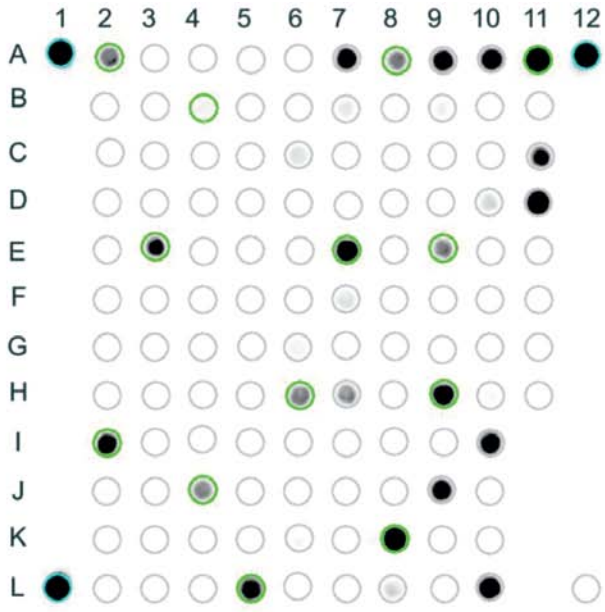
Fig. 1. Schematic diagram of the biochip included in the "NG-TEST" kit and containing 113 immobilized oligonucleotide probes
Рис. 1. Схема биочипа, входящего в набор «NG-ТЕСТ» и содержащего 113 иммобилизованных олигонуклеотидных зондов

the indixes “Ng+” and “Ng–” contain probes for polymorphic loci from the transposable element *ISNgo2* and are used for species identification of *N. gonorrhoeae*. Cells with index “0” do not contain oligonucleotides and are used to normalize the background signal. Cells with index “M” contain a fluorescent marker and are necessary for automatic processing of the biochip hybridization pattern.

The analysis procedure included multiplex amplification and simultaneous fluorescent labeling of *N. gonorrhoeae* genome fragments, followed by hybridization of the resulting products on a biochip, automated registration and interpretation of results using a universal hardware and software complex for biochip analysis (IMB RAS, Russia). Based on the results of the analysis, genetic determinants associated with *N. gonorrhoeae* antimicrobial resistance were determined.

Ceftriaxone MIC value of an individual isolate with an identified set of genetic determinants was calculated using a 20-parameter regression model as described previously [16]. Fig. 2 presents fluorescent patterns and interpretation of the results of analysis using biochips for the purpose of identifying mutations and predicting ceftriaxone MIC. The biochip elements in which the immobilized probes have formed perfect hybridization complexes with the wild-type DNA are highlighted green. The elements of the biochip with complexes of immobilized molecular probes and DNA with mutations are highlighted red (in accordance with the biochip diagram in Fig. 1). Reports on interpretation of the results confirming that the analyzed DNA belongs to the species *N. gonorrhoeae*, the presence/absence of mutations in the *penA*, *ponA* and *porB* genes and the calculated ceftriaxone MIC values when analyzing DNA: 1) an isolate without mutations carrying the non-mosaic *penA* gene (ceftriaxone MIC = 0.002 mg/L); 2) an isolate possessing a mosaic *penA* gene with various substitutions and multiple mutations in the *penA*, *porB*, *ponA* genes (ceftriaxone MIC = 0.031 mg/L). Both isolates are sensitive to ceftriaxone according to the criteria of MUK 4.2.1890-04.

Determination of genetic determinants of resistance of *N. gonorrhoeae* isolates to azithromycin, penicillin, tetracycline, and ciprofloxacin was carried out as described previously [12, 15]. The presence of the C2611T substitution in *23S rRNA* or a genetic profile in which a mosaic allele of the *mtrR* promoter is present with a mosaic allele of *mtrD* were considered as markers of azithromycin resistance. The consistency of this approach was previously shown [21].



Neisseria gonorrhoeae DNA has been detected

No mutations associated with decreased susceptibility to ceftriaxone have been found

Mutations in the *penA gene*:

not found

Mutations in the *ponA gene*:

not found

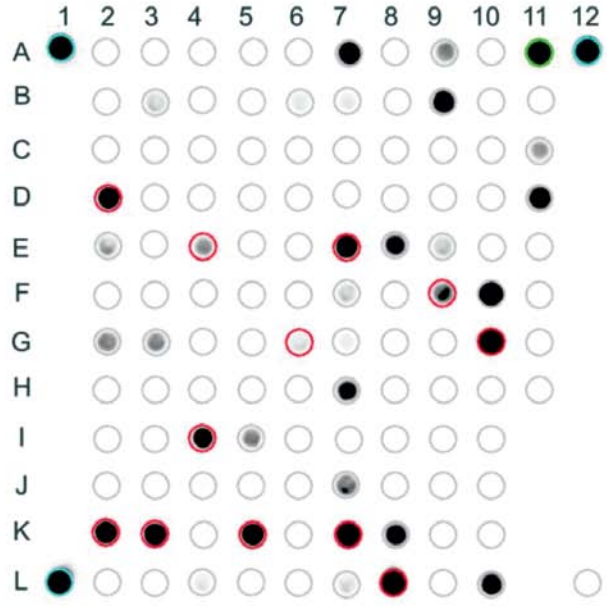
Mutations in the *porB gene*:

120: not found

121: not found

Ceftriaxone MIC (mg/L): 0.002 (< 0.25)

Isolate is susceptible



Neisseria gonorrhoeae DNA has been detected

Mutations associated with decreased susceptibility to ceftriaxone have been found

Mutations in the *penA* gene:
312: Ile->Met (mosaic allele)
316: Val->Pro (mosaic allele)
345-346: ins Asp
483: Thr->Ser (mosaic allele)
551: Pro->Ser

Mutations in the *ponA* gene:
421: Leu->Pro

Mutations in the *porB* gene:
120: Gly->Asp
121: Ala->Gly

Ceftriaxone MIC (mg/L): 0.031 (< 0.25)

Isolate is susceptible

Fig. 2. Fluorescent hybridization patterns and interpretation of the results of biochip-based analysis of *N. gonorrhoeae* isolates using the “NG-TEST” reagent kit
Рис. 2. Флуоресцентные гибридизационные картины и интерпретация результатов анализа изолятов *N. gonorrhoeae* на биочипе с использованием набора реагентов «NG-ТЕСТ»

Statistical analysis

When analyzing the isolates sensitivity to ceftriaxone and azithromycin, the results of resistance determination using a reference microbiological and molecular method were compared and the parameters of diagnostic specificity (S_p) and sensitivity (S_n) were calculated using the following formulas:

$$S_p = T_n / (T_n + F_p) \times 100\ \%,$$
$$S_n = T_p / (T_p + F_n) \times 100\ \%,$$

where T_p and T_n are the true positive and true negative results, respectively; F_p and F_n are the false positive and false negative results, respectively.

Results

Resistance of N. gonorrhoeae isolates to ceftriaxone

All isolates analyzed were sensitive to ceftriaxone; 4.7 % of isolates had a ceftriaxone MIC value of 0.06 mg/L, which is only two dilutions below the resistance threshold. The distribution of genetic determinants of resistance in the population taking into account ceftriaxone MIC is presented in Fig. 3. A significant proportion of isolates with reduced sensitivity (MIC_{cef} > 0.03 mg/L) had substitutions in the *penA* (312Met, 316Thr, 501Val, 545Ser, 551Ser) and *porB* (120Lys) genes. In most cases, it was combinations of mutations in the *penA*, *ponA* and *porB* genes, and not single substitutions, that led to a significant decrease in sensitivity to ceftriaxone. Isolates with reduced sensitivity also exhibited genetic profiles typical for *penA* mosaic alleles, with the following substitutions: Ile312Met, Val316Thr, Asn512Tyr, and Gly545Ser. In total, mosaic alleles of the *penA* gene were found in 4.3 % of the studied sample isolates (see Fig. 3).

The results of comparing ceftriaxone MIC values obtained by a serial dilution method with the MIC value predicted by the "NG-TEST" kit are presented in Table 2. According to the table data, the predicted MIC values coincide well with the experimentally measured ones. For 78 % of the strains, the predicted values differed by not more than one twofold dilution. When analyzing the sample, no false positive results were obtained; all isolates were correctly identified as sensitive ones. Thus, the modern Russian gonococcal population continues to be sensitive to ceftriaxone.

Resistance of N. gonorrhoeae isolates to azithromycin

The proportion of azithromycin-resistant isolates (MIC >1 mg/L) made up a total of 11 % of the entire sample. The distribution of genetic profiles associated with azithromycin resistance when dividing isolates into sensitive and resistant ones is presented in Fig. 4. In azithromycin-resistant isolates, an increase in the proportion of genotypes with a mosaic *mtrR* gene promoter in combination with a mosaic *mtrD* gene was observed. 7.5 % of resistant isolates had C2611T substitutions in the *23S* rRNA gene, and in all cases they were present in all four copies of the rrn operon. No isolates with 2058G or 2059G substitutions in the *23S* rRNA gene were identified. 2.5 % of azithromycin-resistant isolates did not contain mutations in the analyzed *mtrR*, *mtrD* and *23S* rRNA loci. The genetic profile most typical for azithromycin-resistant isolates included the mosaic *mtrD* gene, the mosaic *mtrR* promoter, and a substitution in the coding region of the *mtrR* gene Ala86Thr.

Based on the results of comparing data on phenotypic sensitivity to ceftriaxone and azithromycin and identifying genetic determinants of resistance to these products using microarray technologies, the diagnostic characteristics of molecular methods were determined (Table 3).

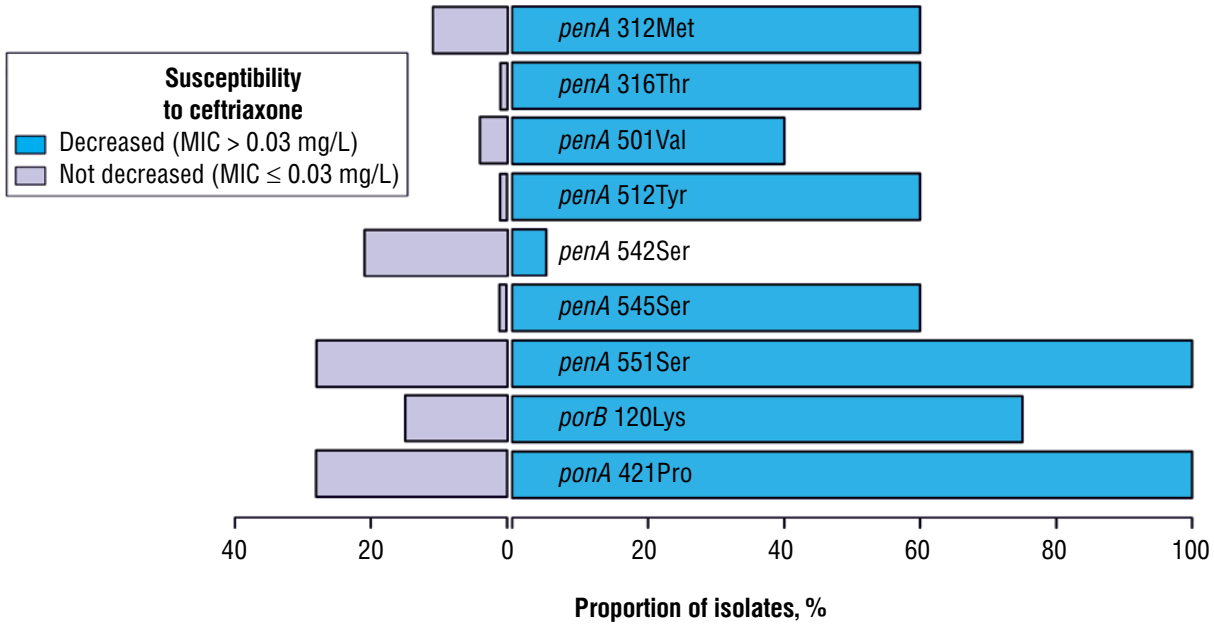


Fig. 3. Distribution of substitutions in the *penA*, *ponA* and *porB* genes when dividing the study sample isolates into groups with reduced (MIC_{cef} > 0.03 mg/L) and non-reduced (MIC_{cef} ≤ 0.03 mg/L) sensitivity to ceftriaxone

Рис. 3. Распределение замен в генах *penA*, *ponA* и *porB* при разделении изолятов исследуемой выборки на группы со сниженной ($МПК_{цеф}$ > 0,03 мг/л) и несниженной ($МПК_{цеф}$ ≤ 0,03 мг/л) чувствительностью к цефтриаксону

Table 2. A comparative analysis of the results obtained for the determination of ceftriaxone MIC in *N. gonorrhoeae* strains, using either a serial dilution method or the NG-TEST kit
Таблица 2. Сравнение результатов определения значения МПК цефтриаксона у штаммов *N. gonorrhoeae*, полученных методом серийных разведений и с использованием набора «NG-ТЕСТ»

Phenotypic data		Predicted MIC_{cef}, mg/L
MIC_{cef}, mg/L	Number of samples	Geometric mean
0.0015	1	0.005
0.002	114	0.0039
0.004	77	0.0077
0.008	76	0.0091
0.015	39	0.0135
0.03	36	0.009
0.06	17	0.0081

Resistance of N. gonorrhoeae isolates to ciprofloxacin, tetracycline and penicillin

Resistance to ciprofloxacin. On average, the proportion of isolates resistant to ciprofloxacin (MIC ≥ 1 mg/L) was 49 %; 1 % were moderately resistant (MIC 0.12–0.50 mg/L) and 50 % were sensitive (MIC ≤ 0.06 mg/L). The distribution of genetic profiles characterizing *N. gonorrhoeae* resistance to ciprofloxacin, when dividing the sample of isolates into sensitive, moderately resistant and resistant ones, is presented in Fig. 5.

In the majority of ciprofloxacin-resistant isolates, mutations in the "quinolone pocket" were detected, among which the Ser91Phe substitution in the *gyrA* gene should be noted. As a rule, in the study sample it was not isolated, but was found in combination with the *gyrA* 95Ala/Gly/Asn and/or *parC* 87Arg/91Gly substitutions, which led to a sharp increase in MIC above the resistance threshold (from 4 to 16 mg/L for the first and second profiles, respectively).

Resistance to penicillin. The proportion of isolates resistant to penicillin (MIC ≥ 2 mg/L) was 8 %; 41 % were moderately resistant (MIC – 0.12–1.00 mg/L), 50 % were sensitive (MIC ≤ 0.06 mg/L). The distribution of genetic profiles characterizing *N. gonorrhoeae* resistance to penicillin, when dividing the sample of isolates into sensitive, moderately resistant and resistant ones, is presented in Fig. 6.

31 % of resistant isolates carried the blaTEM plasmid, the presence of which increased penicillin MIC to 4–32 mg/L. Leu421Pro substitution in the *ponA* gene in combination with mutations in the *penA* and *porB* genes was found in 55 % of resistant isolates. A significant proportion of sensitive isolates had an isolated aspartate insertion at codon 345 of the *penA* gene. The most typical genetic profile for penicillin-

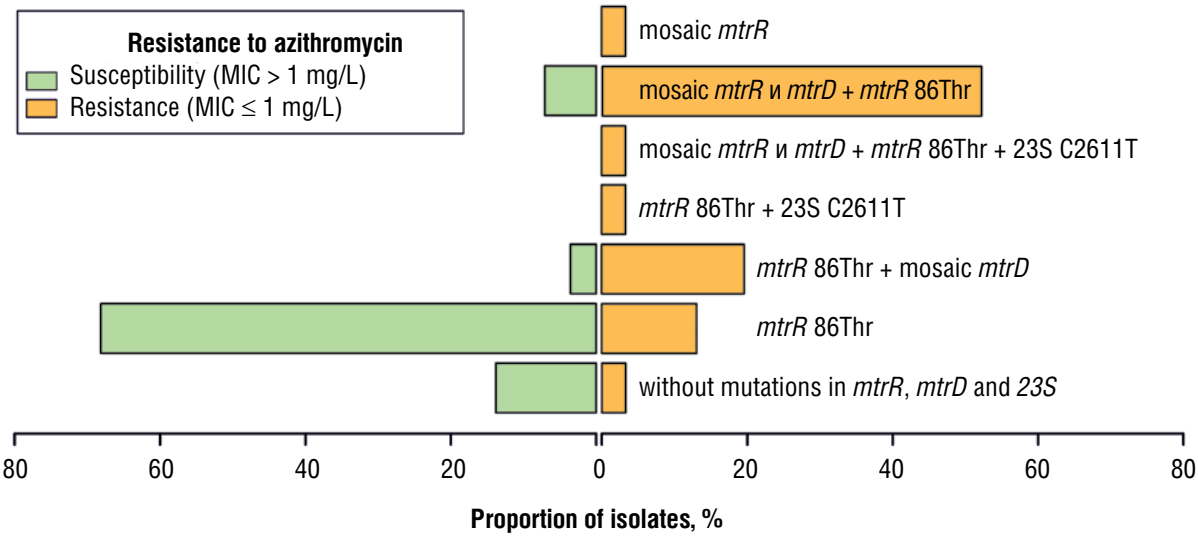


Fig. 4. Distribution of genetic profiles of the *mtrR*, *mtrD*, and 23S rRNA loci in azithromycin-resistant and azithromycin-sensitive isolates in the study sample
Рис. 4. Распределение генетических профилей локусов *mtrR*, *mtrD* и 23S рРНК в устойчивых и чувствительных к азитромицину изолятах в исследуемой выборке

Table 3. A comparative analysis of the results of *N. gonorrhoeae* strains sensitivity to ceftriaxone and azithromycin using the method of serial dilutions and microarray technology
Таблица 3. Сравнение результатов определения чувствительности штаммов *N. gonorrhoeae* к цефтриаксону и азитромицину методом серийных разведений и с использованием микрочиповых технологий

Product	Resistance characteristics	Number of isolates with the corresponding phenotype	Presence of resistance determinants	Sensitivity, %	Specificity, %
Ceftriaxone	Sensitive	360	0	—	100
	Resistant	0	0		
Azithromycin	Sensitive	339	24	88	93
		21	18		

resistant isolates included the Leu421Pro substitution in the *ponA* gene, an aspartate insertion at codon 345 of the *penA* gene with a Gly542Ser substitution in the same gene, and the Gly120Lys mutation in the *porB* gene.

Resistance to tetracycline. On average, the proportion of isolates resistant to tetracycline (MIC ≥ 2 mg/L) was 21 %; 30 % were moderately resistant (MIC – 0.5–1.0 mg/L), 43 % were sensitive (MIC ≤ 0.25 mg/L). The distribution of genetic profiles characterizing *N. gonorrhoeae* resistance to tetracycline, when dividing the sample of isolates into sensitive, moderately resistant and resistant ones, is presented in Fig. 7.

62 % of tetracycline-resistant isolates carried the tetM conjugative plasmid, the presence of which increased tetracycline MIC to 4–16 mg/L. The isolated Ala86Thr mutation in the coding region of the *mtrR* gene was significantly more common in sensitive isolates. The most typical genetic profile for tetracycline-resistant isolates without a conjugative plasmid included the Val57Met substitution in the *rpsJ* gene, an adenine deletion at position 35 of the *mtrR* gene promoter and the Ala86Thr substitution in the same gene, as well as the Gly120Lys mutation in the *porB* gene.

Dynamics of antimicrobial resistance of N. gonorrhoeae isolates in 2019–2023

Analysis of the distribution of genetic profiles characterizing *N. gonorrhoeae* antimicrobial resistance shows different trends in the distribution of typical determinants that make the greatest contribution to the MIC increase. The proportions of isolates with typical genetic determinants of resistance to ciprofloxacin, penicillin, tetracycline and azithromycin in 2019–2023 are presented in Fig. 8.

The disappearance of isolates resistant to penicillin occurs against the background of disappearance of strains with the *blaTEM* plasmid gene in the population (at least in the analyzed sample) and a decrease in the proportion of isolates with chromosomal determinants of resistance — the Leu421Pro substitution in the *ponA* gene and an aspartate insertion in codon 345 of the *penA* gene.

The opposite trend is observed when analyzing tetracycline resistance: an increase in the proportion of

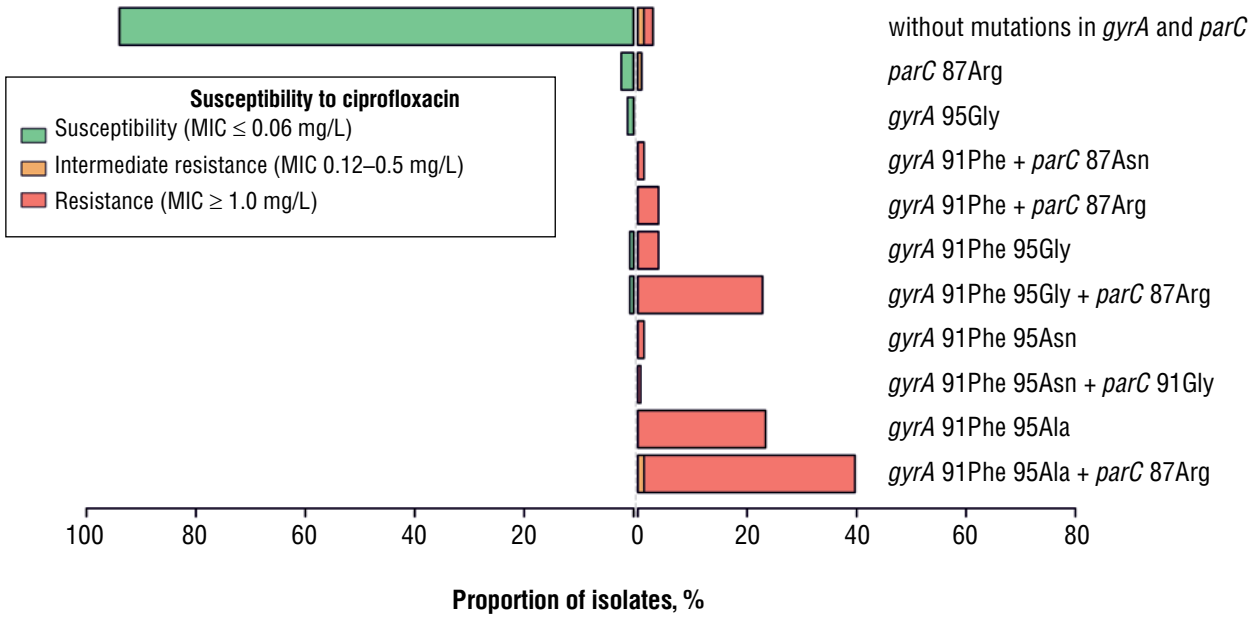


Fig. 5. Distribution of genetic profiles of the *gyrA* and *parC* loci in ciprofloxacin-resistant and ciprofloxacin-sensitive isolates in the study sample
Рис. 5. Распределение генетических профилей локусов *gyrA* и *parC* в устойчивых и чувствительных к ципрофлоксацину изолятах в исследуемой выборке

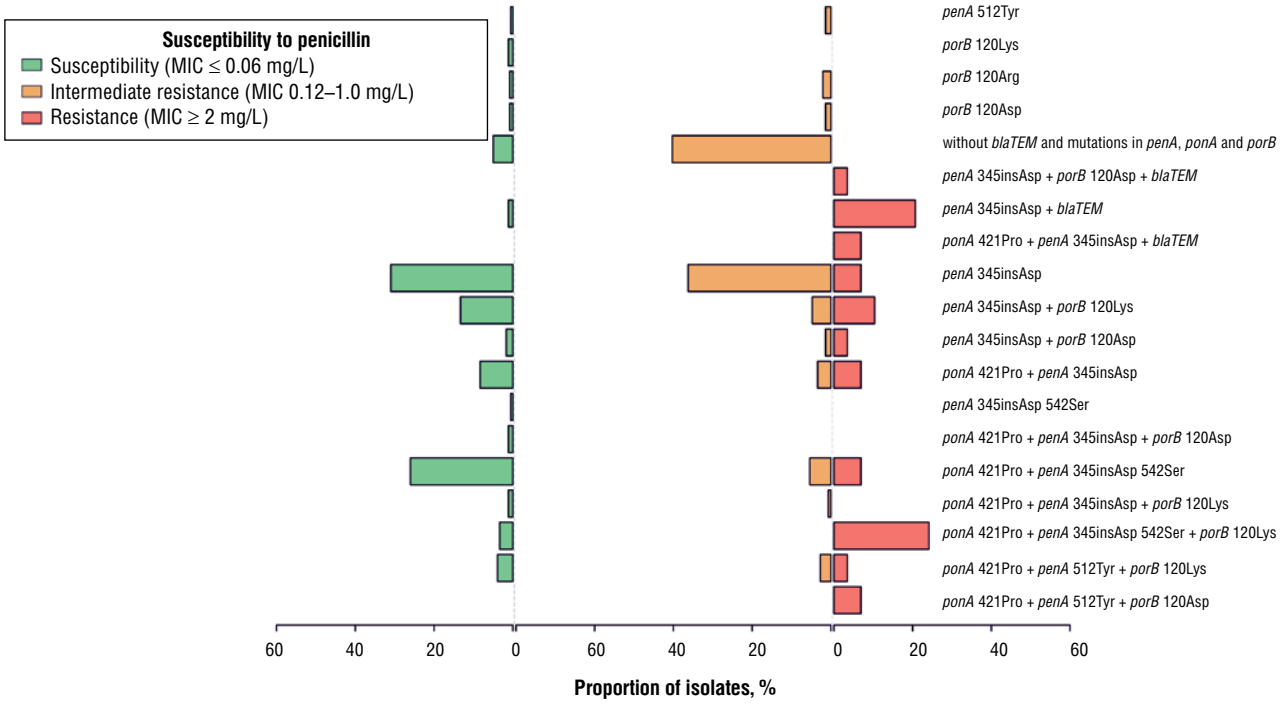


Fig. 6. Distribution of genetic profiles of the *penA*, *ponA*, *porB*, and *blaTEM* loci in penicillin-resistant and penicillin-sensitive isolates in the study sample
Рис. 6. Распределение генетических профилей локусов *penA*, *ponA*, *porB* и *blaTEM* в устойчивых и чувствительных к пенициллину изолятах в исследуемой выборке

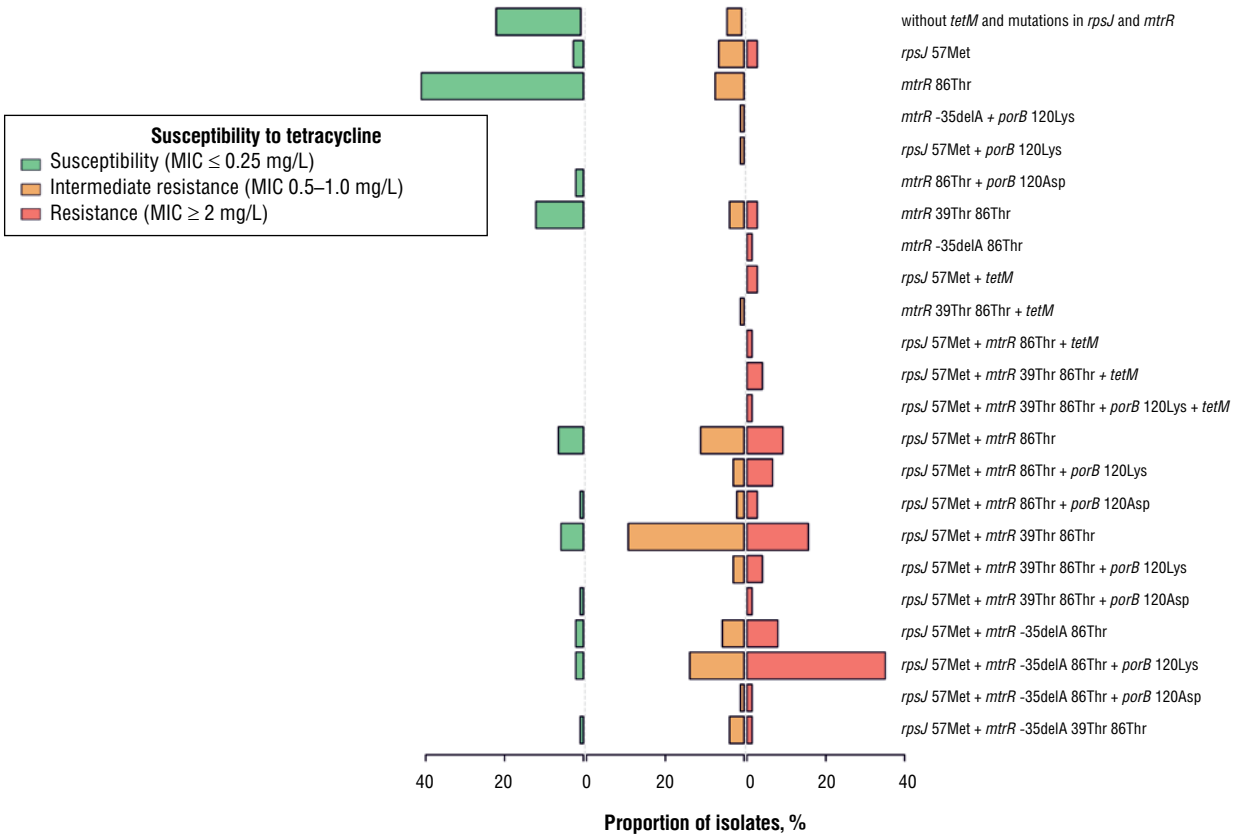


Fig. 7. Distribution of genetic profiles of the *rpsJ*, *mtrR*, *porB*, and *tetM* loci in tetracycline-resistant and tetracycline-sensitive isolates in the study sample
Рис. 7. Распределение генетических профилей локусов *rpsJ*, *mtrR*, *porB* и *tetM* в устойчивых и чувствительных к тетрациклину изолятах в исследуемой выборке

isolates with chromosomal determinants localized in the *rpsJ*, *mtrR* and *porB* genes is recorded. Of particular note is the preservation of strains with the *tetM* plasmid gene, the most "powerful" determinant of tetracycline resistance. In general, the proportion of tetracycline-resistant isolates remains at a consistently high level (~30 %).

The annual increase in the proportion of ciprofloxacin-resistant isolates is confirmed by the corresponding determinants in the *gyrA* and *parC* genes, including the Ser91Phe substitution in *gyrA*. There is currently no trend towards a decrease in *N. gonorrhoeae* resistance to fluoroquinolones in Russia.

In 2019, no azithromycin-resistant isolates were identified. Their appearance dates back to 2020, with the consolidation of strains possessing a mosaic promoter of the *mtrR* gene with the mosaic *mtrD* gene in the Russian gonococcal population. At the same time, isolates with mutations in the *23S* rRNA gene remain rare: during the analysed period, only three isolates with the C2611T polymorphism were identified.

Discussion

The results of this study show that gonococcal ceftriaxone resistance in Russia meets the WHO criteria for the use of an antibiotic (the proportion of sensitive isolates in the population is more than 95 %). At the same time, almost 5 % of identified isolates with reduced ceftriaxone sensitivity (MIC > 0.03 mg/L) and the spread of resistant strains around the world, along with the use of ceftriaxone as the antimicrobial drug of choice, determine the importance of using molecular methods to analyze *N. gonorrhoeae* resistance to this product.

Since 2020, the emergence and consolidation of azithromycin-resistant isolates in the Russian gonococcal

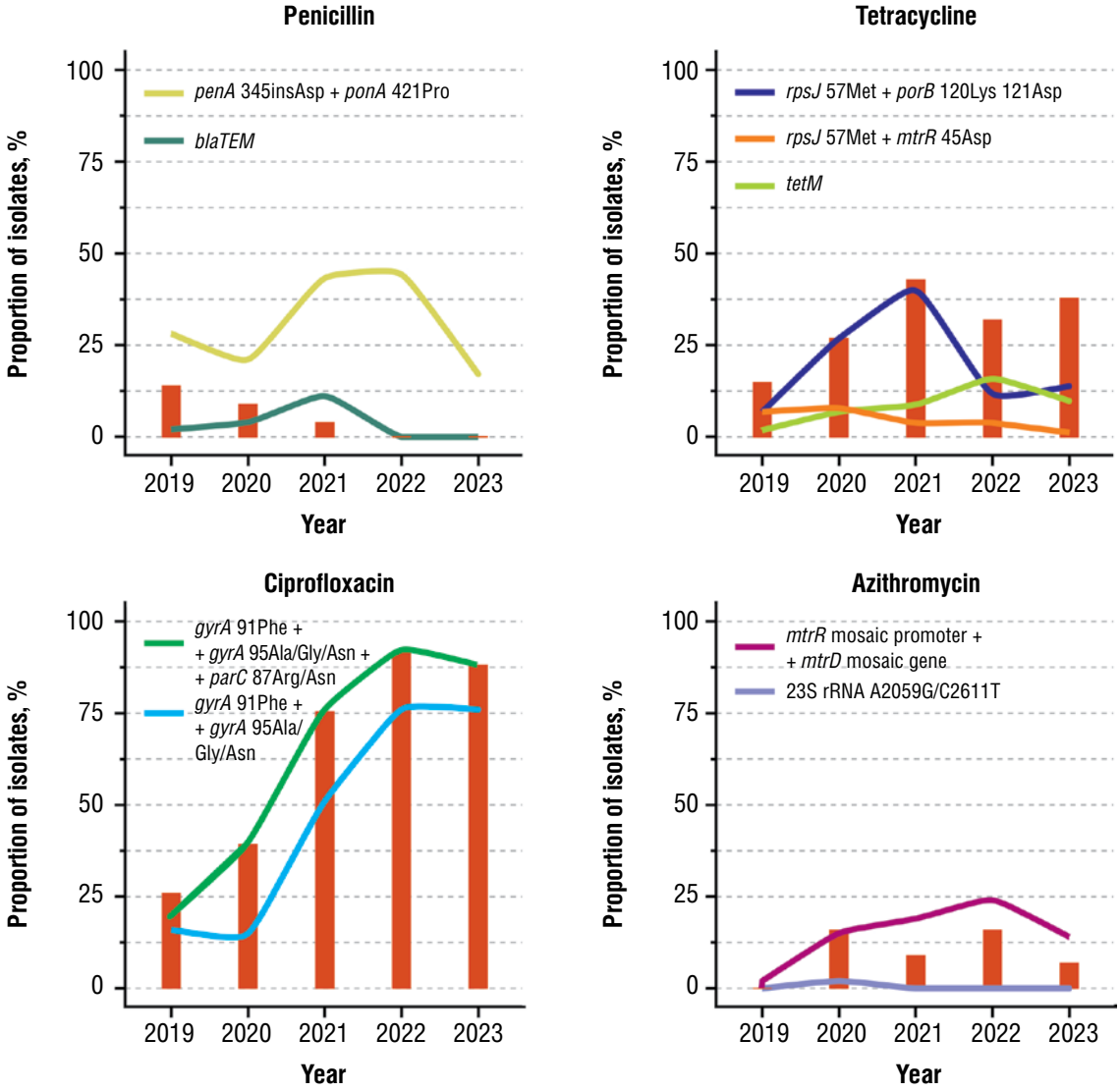


Fig. 8. Dynamics of the incidence in 2019–2023 of *N. gonorrhoeae* isolates resistant to penicillin, tetracycline, ciprofloxacin, azithromycin, possessing the genetic determinants that make the greatest contribution to the MIC increase. The lines indicate the change in the proportions of characteristic genetic determinants. The histogram columns reflect the annual proportion of isolates with phenotypic resistance to the corresponding antimicrobial drug

Рис. 8. Динамика встречаемости в 2019–2023 гг. изолятов *N. gonorrhoeae*, устойчивых к пенициллину, тетрациклину, ципрофлоксацину, азитромицину, обладающих генетическими детерминантами, вносящими наибольший вклад в повышение МПК. Линиями обозначено изменение долей характерных генетических детерминант. Столбцы гистограммы отражают ежегодную долю изолятов с фенотипической устойчивостью к соответствующему противомикробному препарату

population, previously found only sporadically, have been registered [22]. Due to the significant proportion of such isolates exceeding the WHO criterion, the advisability of using this product for the gonococcal infection treatment in the general case has been questioned.

In a study of isolates obtained in Russia in 2005–2016, a trend towards a decrease in resistance to penicillin, tetracycline and ciprofloxacin was observed, and it was associated with the exclusion of these products from therapy regimens [22]. The results of this work show that this trend remains only for penicillin. There is still a proportion of moderately resistant strains (or strains that are resistant at increased exposure) while isolates with a true penicillin-resistant phenotype are reduced and eliminated. An interesting feature of the 2022–2023 population was a very small proportion of isolates with the *blaTEM* plasmid gene, while isolates with the *tetM* plasmids were retained. It has previously been shown that the *tetM* conjugative plasmid in *N. gonorrhoeae* can facilitate the transfer of other plasmids, which are often found with the *tetM* [24] plasmids, into the cell, including *blaTEM* [23]. Despite the discontinuation of using ciprofloxacin for the treatment of gonorrhea, an increase in isolates with multiple resistance determinants in the "fluoroquinolone pocket" (in the *gyrA* and *parC* genes) and the persistence of a high proportion of ciprofloxacin-resistant isolates have been observed. In the absence of selective product pressure, these mutations should negatively impact bacterial fitness, but this process is paradoxically not accompanied by rapid elimination of the corresponding variants from the *N. gonorrhoeae* population.

The developed microarray technologies have been validated using 360 clinical isolates. The diagnostic specificity of the "NG-TEST" reagent kit was 100 %, and no false negative results were obtained. A distinctive feature of the created method is not just the separation of isolates into sensitive and resistant ones, but also the determination of ceftriaxone MIC value for the analyzed sample. In the present study, the method showed good repeatability with the phenotypic determination of MIC for 78 % of the isolates. In addition to predicting MIC, the developed method provides data on the presence of genetic determinants of drug resistance to cephalosporins, and therefore it is suitable for solving problems of both clinical laboratory diagnostics and molecular epidemiology of gonococcus.

When determining azithromycin resistance using a biochip, diagnostic sensitivity and specificity values of 88 and 93 %, respectively, were obtained. These characteristics are comparable to the results obtained from analysis of data on the whole genome sequencing of azithromycin-resistant *N. gonorrhoeae* isolates. According to the Pathogenwatch database, the sensitivity and specificity of this method are 72 and 100 %, respectively. [25]

The previously described biochips, [12, 14, 15] which allow to obtain data on the determinants of *N. gonorrhoeae* resistance to previously used ciprofloxacin, penicillin and tetracycline, are also of interest for molecular epidemiology. When analyzing the gonococcal population, it was shown that for predicting the sensitivity of an isolate to antimicrobial drugs, it is necessary to take into account the genetic profile of mutations in various loci, while the contribution of each mutation to phenotypic sensitivity is generally different. The use of microarray technologies will allow in the future to carry out the dynamic monitoring of *N. gonorrhoeae* transmission routes in the regions, improve the epidemiological surveillance system and increase the efficacy of gonorrhea treatment.

Conclusion

In the Russian *N. gonorrhoeae* population, active processes are occurring that are associated with the redistribution of the proportions of isolates resistant to various antimicrobial drugs. Since 2020, azithromycin resistance has increased sharply, the proportion of ciprofloxacin-resistant isolates is growing, penicillin sensitivity has been restored, but the entire population remains sensitive to ceftriaxone.

The "NG-TEST" reagent kit, developed and validated with 360 samples, ensures rapid determination of *N. gonorrhoeae* resistance to ceftriaxone through simultaneous identification of genetic determinants of resistance in the *penA*, *ponA* and *porB* genes and calculation of the MIC value. This method and other microarray technologies for identification of the determinants of *N. gonorrhoeae* antimicrobial resistance can be used as an auxiliary tool for identifying resistant *N. gonorrhoeae* strains. The use of microarray technologies will facilitate both the selection of the correct treatment strategy for a particular patient and the collection of epidemiological information, providing the possibility to monitor the molecular epidemiological picture at the population level.

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