JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3681–3689 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.00565-10


# Molecular Surveillance of Clinical *Neisseria gonorrhoeae* Isolates in Russia[▽]†

Elena N. Ilina,* Nina Y. Oparina, Egor A. Shitikov, Alexandra D. Borovskaya, and Vadim M. Govorun

*Research Institute for Physico-Chemical Medicine of Ministry of Public Health of Russian Federation, Moscow, Russia*

Received 16 March 2010/Returned for modification 28 April 2010/Accepted 12 July 2010

**The choice of adequate methods for epidemiological purposes remains a challenging problem in *Neisseria gonorrhoeae* molecular monitoring. In this study, the collection of geographically unrelated gonococci ($n = 103$) isolated in Russian clinics was comparably tested by (i) a traditional serotyping scheme, (ii) *por* typing, (iii) *Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST), and (iv) multilocus sequence typing (MLST). It is shown that, according to sequencing data, a third of the strains carried new *porB1* alleles, as well as *tbpB* ones, and more than half of the samples had new sequence types (STs) as determined by NG-MAST or MLST. The discriminatory power for each typing method was calculated by using the Hunter-Gaston discriminatory index, *D*. Commonly, modern nucleic acid-based typing methods (*por* typing, NG-MAST, and MLST) appeared to be more efficient than the classical serotyping scheme. While the traditional serotyping gave a *D* value of 0.82, the *por* typing, NG-MAST, and MLST approaches yielded *D* values of 0.97, 0.98, and 0.91, respectively. Each typing technique revealed the distribution of gonococci slightly correlated with their geographical sources. However, only the MLST method STs were highly associated with certain phenotypes. Although ST1594, ST1892, and ST6720 were typical for susceptible gonococci, ST1901 and ST6716 were undoubtedly associated with a multidrug-resistant phenotype. We conclude that every tested nucleic acid-based typing method is suitable for *N. gonorrhoeae* molecular surveillance. However, the MLST method seems to serve large-scale epidemiological purposes, whereas the NG-MAST and *por* typing approaches are more appropriate for the investigation of local outbreaks.**

The choice of an optimal epidemiological approach for *Neisseria gonorrhoeae* typing remains a problem for public health control strategies. The worldwide spread of drug-resistant strains requires typing methods to be introduced into a national surveillance programs that are already realized within the Australian Gonococcal Surveillance Programme (33). In Russia, strains that are resistant to penicillin, tetracycline, and fluoroquinolones are common, reaching up to 60% prevalence in some regions (14). As is generally known, most resistance mechanisms in *N. gonorrhoeae* are linked to mutations in genomic DNA, and a wide dissemination of such mutations has been demonstrated in subjects with gonorrhea in the Russian population (11). In fact, chromosomally mediated drug resistance expands clonally in a bacterial population. Thus, tracing the spread of gonorrhea by using strain differentiation methods is of great importance. In addition, population genetics are important in understanding the evolutionary history, epidemiology, and population dynamics of pathogens.

Widespread adoption of the molecular diagnosis of gonorrhea could compromise traditional bacteriological cultivation in routine practice and highlights the need for the development of molecular tools for *N. gonorrhoeae* typing. Several promising approaches exist in this field. One of them is *por* typing (7, 8, 27), based on comparative analysis of *porB* gene nucleotide sequences. It possesses rather high discriminatory power (39) and presents data in a format comparable with serotyping data. It should be mentioned that PorB1 protein plays a key role in the adhesion of gonococci to epitheliocytes and reflects the pathogenicity of isolates. Moreover, in earlier studies the multidrug-resistant phenotype of *N. gonorrhoeae* was bound to certain serotypes determined in accordance with the antigenic properties of PorB1 proteins (7, 21, 29).

Today, the *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) (19) appears to be a leading method for *N. gonorrhoeae* typing. It is based on the analysis of internal fragments of two hypervariable genes, *porB* and *tbpB*, encoding superficial gonococcus antigens and therefore under positive selection (19). An online database (www.ng-mast.net) collects current information on the sequence types of gonococcus isolates in different regions of the world, and it can be an efficient instrument for objective estimation of the genetic variability of the microbial population and tracing the spread of infection (2). Moreover, an association of the NG-MAST sequence type with the antibiotic resistance profile has been demonstrated by some investigators (20, 26).

In addition, NG-MAST, which is usually carried out on DNA extracted from a pure bacterial culture, can be performed directly from noncultured samples such as a piece of clothing (18) or clinical specimens (40). Although some researchers have reported a successful application of NG-MAST to urogenital specimens (urine specimens and swabs from the cervix, urethra, and vagina) and to rectal swabs, this typing method was found to be less suitable for throat swabs due to cross-reaction with commensal *Neisseria* species. It seems that direct typing schemes utilizing probe hybridization methods

* Corresponding author. Mailing address: Research Institute of Physicochemical Medicine, Malaya Pirogovskaya St., 1a, 119435, Moscow, Russia. Phone: 7-499-2450471. Fax: 7-499-2467721. E-mail: ilinaen@gmail.com.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 21 July 2010.

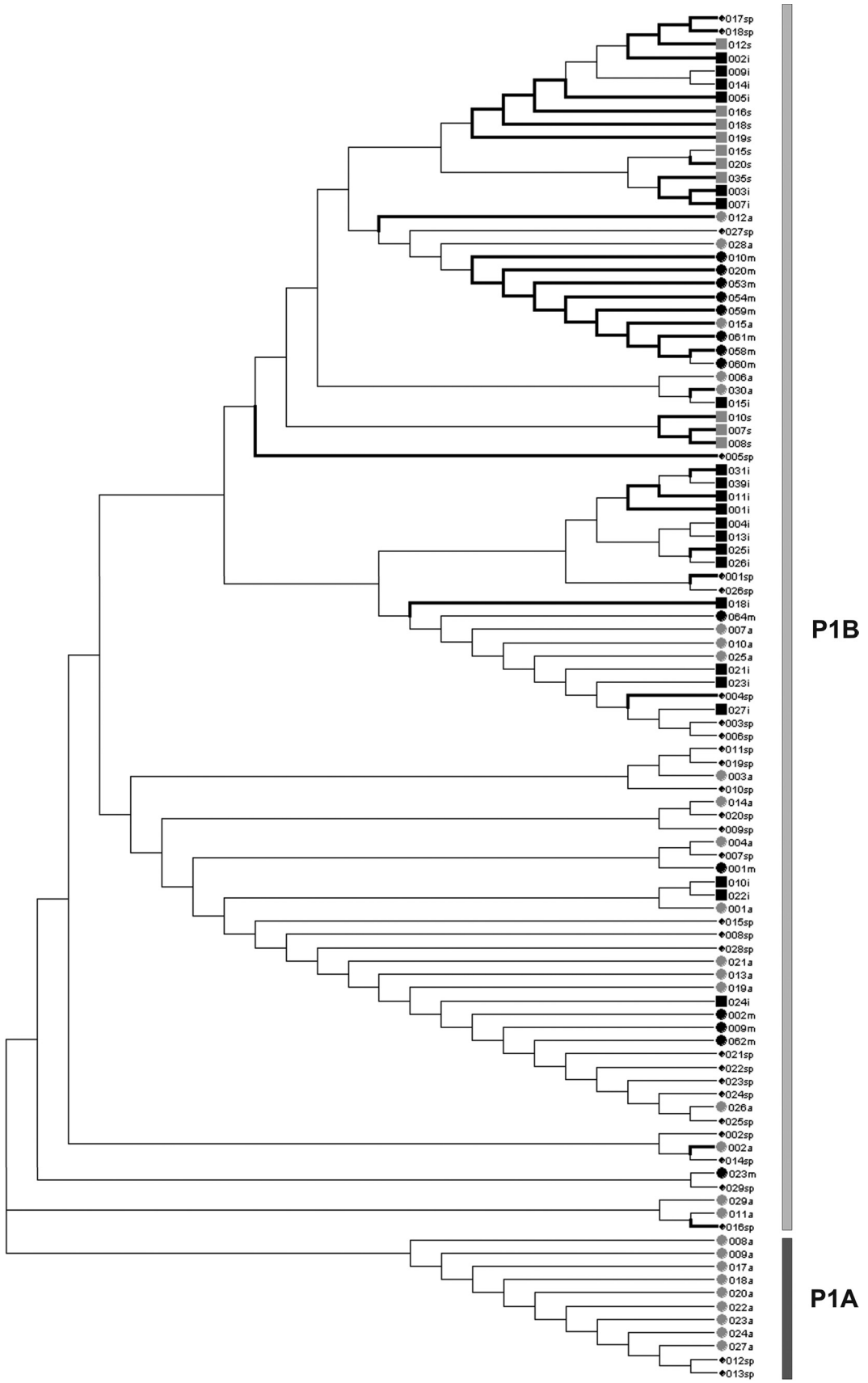



FIG. 1. Phylogenetic tree based on analysis of the entire *porB1* gene nucleotide sequences. Serovars P1A and P1B are indicated by vertical lines. Clinical *N. gonorrhoeae* strains ($n = 103$) are named according to Table S2 in the supplemental material. Isolate origins: Irkutsk ($n = 22$), black square; Samara ($n = 10$), gray square; Murmansk ($n = 14$), large black circle; St. Petersburg ($n = 29$), small black circle; Arkhangelsk ($n = 28$), large gray circle. Multiresistant isolates (i.e., isolates resistant to at least two antibiotics) are indicated by thick lines.

TABLE 1. Variability of entire *porB* gene nucleotide sequences for 103 *N. gonorrhoeae* clinical isolates

| Locus | Length (bp) | No. of: | | | Nucleotide variability ($\pi$) | Z-test result[a] | | |
|---|---|---|---|---|---|---|---|---|
| | | Isolates | Alleles | Polymorphic sites | | $dN < dS$ | $dN = dS$ | $dN > dS$ |
| *porB* | 933–1020 | 103 | 58 | 257 | 0.062 | 0.0013 | 0.0032 | 1.0 |
| *porB1a* | 933 | 11 | 2 | 18 | 0.004 | 1.0 | 0.0076 | 0.0038 |
| *porB1b* | 990–1020 | 92 | 56 | 135 | 0.030 | 0.21 | 0.44 | 1.0 |

[a] A Z-test was performed to check the null hypothesis as to whether the *porB* allele is under purifying selection ($dN < dS$), diversifying positive selection ($dN > dS$), or neutral selection ($dN = dS$). *dN*, nonsynonymous substitutions; *dS*, synonymous substitutions.

(16, 17) for a broad spectrum of clinical samples are more convenient than sequencing.

The opposite approach—analysis of conservative, presumably selective neutral housekeeping genes—is taken by multilocus sequence typing (MLST) (1, 38). Possessing a sufficient number of allelic variants and characterized by the slow accumulation of mutations, these genes reflect the natural evolution of the microbial population and common trends in the spread of gonorrhea.

Often, the effectiveness of *N. gonorrhoeae* surveillance depends on the methods used for species identification and the typing of clinical isolates. However, considering the variety of typing systems but the lack of world experience in their application to monitoring gonococci, it has not yet been determined whether there is a unique objective method for revealing the relationships between clinical isolates. The goal of the present study was to evaluate a number of approaches, including *por* typing, NG-MAST, MLST, and serotyping for typing geographically unrelated gonococcal isolates in Russia.

(This study was partially presented during the 16th International Pathogenic *Neisseria* Conference in 2008 in the Netherlands.)

## MATERIALS AND METHODS

**Bacterial isolates.** *N. gonorrhoeae* clinical isolates collected in different regions of Russia from 2004 to 2005 were obtained from the Central Research Institute of Dermatology and Venereology. All isolates were identified as *N. gonorrhoeae* and tested for serotype and for susceptibility to penicillin G (PEN), tetracycline (TET), ciprofloxacin (CIP), spectinomycin, and ceftriaxone as a part of a previous investigation (11).

**Genetic analysis.** Genomic DNA from *N. gonorrhoeae* was extracted according to the method of Boom et al. (4). When necessary, the prepared DNA samples were stored at −20°C.

Amplification reactions were carried out in 10 μl of 66 mM Tris-HCl (pH 9.0), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primers (see Table S1 in the supplemental material), and 1 U of *Taq* polymerase (Fermentas, Lithuania) under the following conditions: 35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 15 s. A programmed thermocycler Tetrad DNA Engine (MJ Research, Inc.) was used.

Dephosphorylation of the 5′-end phosphate groups of dNTPs and cleavage of primers in the postamplification reaction mixture was done by incubation with 0.5 U of shrimp alkaline phosphatase and 0.1 U of exonuclease I (both enzymes from Fermentas, Lithuania) for 20 min at 37°C, followed by the inactivation by heating for 10 min at 85°C.

Sequencing procedure was performed by the modified Sanger method using a ABI Prism BigDye terminator cycle sequencing ready reaction kit and an ABI Prism 3100 genetic analyzer (Applied Biosystems, Hitachi, Japan) according to the manufacturer's instructions.

Analysis of the nucleotide sequences was carried out by using Vector NTI Advance v.9.0 software (Infomarks, Inc.). The DNA sequences of *porB* and *tbpB* genes collected during the present study were submitted to GenBank (accession numbers EU530732 to EU530817 and EU532618 to EU532759, respectively). The DNA sequences of housekeeping genes were uploaded on the http://pubmlst.org website.

**NG-MAST or MLST.** Characterization of all isolates by NG-MAST and MLST was performed as originally described (1, 19). The corresponding sequences were submitted to the NG-MAST (http://www.ng-mast.net) or MLST (http://pubmlst.org/neisseria) websites for comparison with the existing alleles for determination of the allele types and sequence types (STs) of the isolates.

**Data analysis.** Sequences were aligned by using CLUSTAL X 1.8 software (http://www.clustal.org). At the next step, aligned nucleotide sequences were converted to MEGA version 4.0.2 software (http://www.megasoftware.net), and distance matrices were estimated (using the nucleotide Tajima-Nei model). Further classification was constructed by using the neighbor-joining method based on a bootstrap tree stability test (500 iterations).

Visual mapping of multidrug resistance and place of strain isolation were carried out by using the Dendroscope tree editor (http://www-ab.informatik.uni-tuebingen.de/software/dendroscope/welcome.html) (10).

The discriminatory power for each typing method was defined by calculation of a single numerical index of discrimination (i.e., the Hunter-Gaston discriminatory index [*D*]) (9), based on the probability that two unrelated isolates would be placed into different typing groups:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

where $N$ is the number of unrelated strains tested, $S$ is the number of different types, and $n_j$ is the number of strains belonging to the $j$th type.

The calculated data was interpreted with commonly accepted decision that a *D* of >0.90 is desirable for a confident typing method (9).

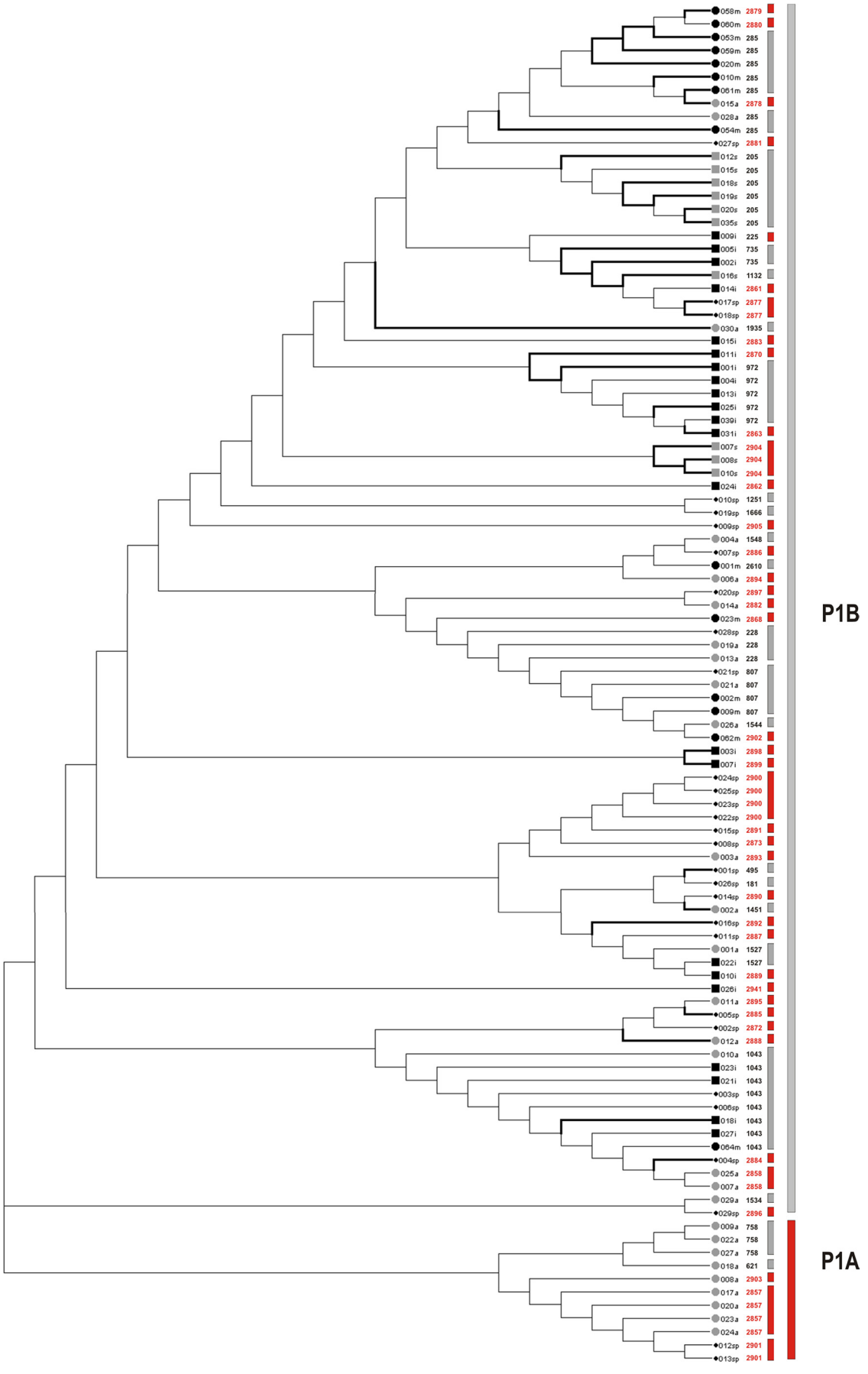
A recombination test to determine the index of association and the standardized index of association ($I_A$ and $I^S_A$, respectively) was performed using the START2 program (12). Allele profile data were analyzed in eBURST (version 3; http://eburst.mlst.net/v3/enter_data/single/) to define clonal complexes or groups (6).

Associations between sequence type and susceptibility level were examined by using chi-square ($\chi^2$) and Cramer (V) tests. All statistical calculations were two-tailed and were conducted with a significance level of at least $P < 0.05$. Statistical analysis was performed with Statistica 7.0 software.

## RESULTS

In all, 103 clinical *N. gonorrhoeae* isolates from five remote regions of Russia—Irkutsk ($n = 22$), Samara ($n = 10$), Arkhangelsk ($n = 28$), Murmansk ($n = 14$), and St. Petersburg ($n = 29$)—were investigated (see Table S2 in the supplemental material).

**Protein-based typing method.** The traditional serotyping procedure divided all typing isolates into 11 different groups, with a prevalence of the PIB serovar (92/103 [89.3%]). Within this serovar the PIB2, PIB3, PIB5, and PIB22 serotypes were detected in 30 (29.1%), 27 (26.2%), 10 (9.7%), and 10 (9.7%) cases, respectively. The remaining PIB serovar isolates ($n = 15$) belonged to five different serotypes: PIB3/6 ($n = 4$), PIB4 ($n = 4$), PIB8 ($n = 2$), PIB9 ($n = 3$), and PIB26 ($n = 2$). Among the PIA serovar isolates, the PIA6 serotype was found


058m 2879
060m 2880
053m 285
059m 285
020m 285
010m 285
061m 285
015a 2878
028a 285
054m 285
027sp 2881
012s 205
015s 205
018s 205
019s 205
020s 205
035s 205
009i 225
005i 735
002i 735
016s 1132
014i 2861
017sp 2877
018sp 2877
030a 1935
015i 2883
011i 2870
001i 972
004i 972
013i 972
025i 972
039i 972
031i 2863
007s 2904
008s 2904
010s 2904
024i 2862
010sp 1251
019sp 1666
009sp 2905
004a 1548
007sp 2886
001m 2610
006a 2894
020sp 2897
014a 2882
023m 2868
028sp 228
019a 228
013a 228
021sp 807
021a 807
002m 807
009m 807
026a 1544
062m 2902
003i 2898
007i 2899
024sp 2900
025sp 2900
023sp 2900
022sp 2900
015sp 2891
008sp 2873
003a 2893
001sp 495
026sp 181
014sp 2890
002a 1451
016sp 2892
011sp 2887
001a 1527
022i 1527
010i 2889
026i 2941
011a 2895
005sp 2885
002sp 2872
012a 2888
010a 1043
023i 1043
021i 1043
003sp 1043
006sp 1043
018i 1043
027i 1043
064m 1043
004sp 2884
025a 2858
007a 2858
029a 1534
029sp 2896
009a 758
022a 758
027a 758
018a 621
008a 2903
017a 2857
020a 2857
023a 2857
024a 2857
012sp 2901
013sp 2901
P1B
P1A

in 10 (9.7%) isolates, and one sample belonged to the PIA10 serotype. The calculated *D* value was found to be 0.82, which shows that the discriminatory power of serotyping is not sufficient to make it an effective typing method.

**Nucleic acid-based typing methods.** Nucleic acid-based techniques, such as *por* typing, NG-MAST, and MLST, divided all of the isolates into 58, 61, and 30 groups, respectively.

***por* typing.** Alignment of the entire *porB* gene nucleotide sequences revealed the sufficient heterogeneity of *N. gonorrhoeae* clinical isolates. The dendrogram constructed by the neighbor-joining algorithm is shown in Fig. 1. When a cutoff value of 0.1 (genetic distance) was used, 58 clusters could be delineated. Expectedly, all gonococcal isolates were separated into two large groups corresponding to the *porB1a* ($n = 11$) and *porB1b* ($n = 92$) alleles of the *porB* gene. Comparative analysis of the nucleotide polymorphism showed a much higher variability for the *porB1b* allele than for *porB1a*. Variability for the *porB1b* allele became apparent in a number of exposed allelic variants and polymorphic sites within each group. Moreover, 3-, 6-, or 12-nucleotide insertions, as well as 6-nucleotide deletions, were found for several isolates possessing the *porB1b* allele. Analysis of the nonsynonymous/synonymous substitution rate ratio revealed different trends in the natural selection of *porB* alleles (Table 1). While the influence of positive diversifying selection was shown for the *porB1b* allele, the *porB1a* allele seemed to be under the influence of purifying selection. Commonly, the discriminatory index for this typing method was 0.97, which is sufficient for epidemiological purposes.

Comparative analysis of internal fragments of *porB* genes (490 bp) via the public database on the NG-MAST website (http://www.ng-mast.net) showed that a third of tested group carried new *por* alleles unrepresented in the nucleotide library. The 49 isolates formed six clusters carrying *por-37* ($n = 10$), *por-90* ($n = 10$), *por-91* ($n = 8$), *por-164* ($n = 6$), *por-232* ($n = 7$), and *por-685* ($n = 8$) alleles. Among these, the *por-90* cluster was clearly associated with the PIA6 serotype, and the *por-91*, *por-164*, and *por-232* clusters contained gonococci isolated in Irkutsk, Samara, and Murmansk, respectively.

**NG-MAST.** Based on analysis of *porB* and *tbpB* fragments, all isolates were characterized by NG-MAST. We found 51 and 27 variants of *porB* and *tbpB* alleles, respectively, which resulted in the assignment of 61 different sequence types (STs). A phylogenetic tree based on the concatenated sequences of two loci is shown in Fig. 2. The 57 (55%) isolates belonged to 15 different STs containing from two to eight members. The largest of them were ST1043 ($n = 8$), ST285 ($n = 7$), ST206 ($n = 6$), and ST972 ($n = 5$). The other 46 STs were represented by individual isolates, including four penicillinase-producing *N. gonorrhoeae* (PPNG) isolates collected in St. Petersburg ($n = 3$) and Murmansk ($n = 1$). Meanwhile, approximately half of the isolates ($n = 50$) belonged to the 39 new STs described here for the first time. Generally, there was no clear geographic clustering of specific NG-MAST types found within the country. In some cases, certain STs were found for gonococci isolated in the same place, for example, ST205 in Samara, ST285 in Murmansk, and ST972 in Irkutsk. However, this probably does not reflect local outbreaks but rather the global spread of infection. The calculated index of discrimination for this typing method was 0.98, which reflected the strong discriminatory power of NG-MAST.

**MLST.** According to MLST data, the 30 different groups (STs) were discovered. Among them, 13 ST clusters of between 2 and 25 isolates were identified. ST6716 ($n = 25$) were found to be the most prevalent, the other largest groups were ST1901 ($n = 11$), ST1905 ($n = 11$), ST1892 ($n = 8$), ST1584 ($n = 8$), and ST6720 ($n = 5$). The 17 STs were represented by single isolates. Concatenated sequences of seven housekeeping gene fragments were analyzed by using START 2.0 software. A phylogenetic tree constructed by using the neighbor-joining method is shown in Fig. 3. The calculated *D* value was determined to be 0.91 by MLST, which demonstrates this method's applicability as an effective typing method.

Analysis by eBURST showed that four nonoverlapping groups (clonal complexes) contained STs, which matched at least one other ST at five or more loci (Fig. 4). Four STs—ST1901, ST1927, ST6716, and ST6715—were recognized as hyperinvasive genotypes. Among them, ST1901 and ST6716 were evolutionary related, sharing six of seven alleles.

Based on MLST data, the examined *N. gonorrhoeae* isolates were compared to gonococcal isolates collected in the United Kingdom (1) in terms of allelic diversity (Table 2) and recombination parameters (Table 3). The most changeable loci were found to be similar to these collections, and significant linkage disequilibrium was detected for both populations.

**Genotyping data and antimicrobial resistance.** *N. gonorrhoeae* clinical isolate distribution according to different nucleic acid-based typing schemes (*por* typing, NG-MAST, and MLST) was compared to their susceptibility profiles. Since many STs were represented by occasional isolates, only clusters that contained five or more members were considered. Among them, NG-MAST ST205 ($n = 6$), ST285 ($n = 7$), and ST282 ($n = 5$) were unambiguously associated with multidrug resistance, i.e., intermediate susceptibility or resistance to penicillin, tetracycline and ciprofloxacin. NG MAST ST1043 ($n = 8$) included gonococci not susceptible to PEN, resistant to TET, and susceptible to CIP. Appropriately, the same isolates also formed corresponding clusters in accordance with the *por* typing results.

A relationship between the *N. gonorrhoeae* phenotype and certain STs was observed by MLST (Table 4). Although ST1594, ST1892, and ST6720 were typical for susceptible gonococci, ST1901 and ST6716 were undoubtedly associated with multidrug resistance. ST1905 comprised isolates display-

FIG. 2. Phylogenetic tree based on analysis of concatenated sequences of *porB1* and *tbpB* gene fragments (NG-MAST scheme). Newly described STs are indicated in red; serovars P1A and P1B are indicated too. Clinical *N. gonorrhoeae* strains ($n = 103$) are named according to Table S2 in the supplemental material. Isolate origins: Irkutsk ($n = 22$), black square; Samara ($n = 10$), gray square; Murmansk ($n = 14$), large black circle; St. Petersburg ($n = 29$), small black circle; Arkhangelsk ($n = 28$), large gray circle. Multiresistant isolates (i.e., isolates resistant to at least two antibiotics) are indicated by thick lines.

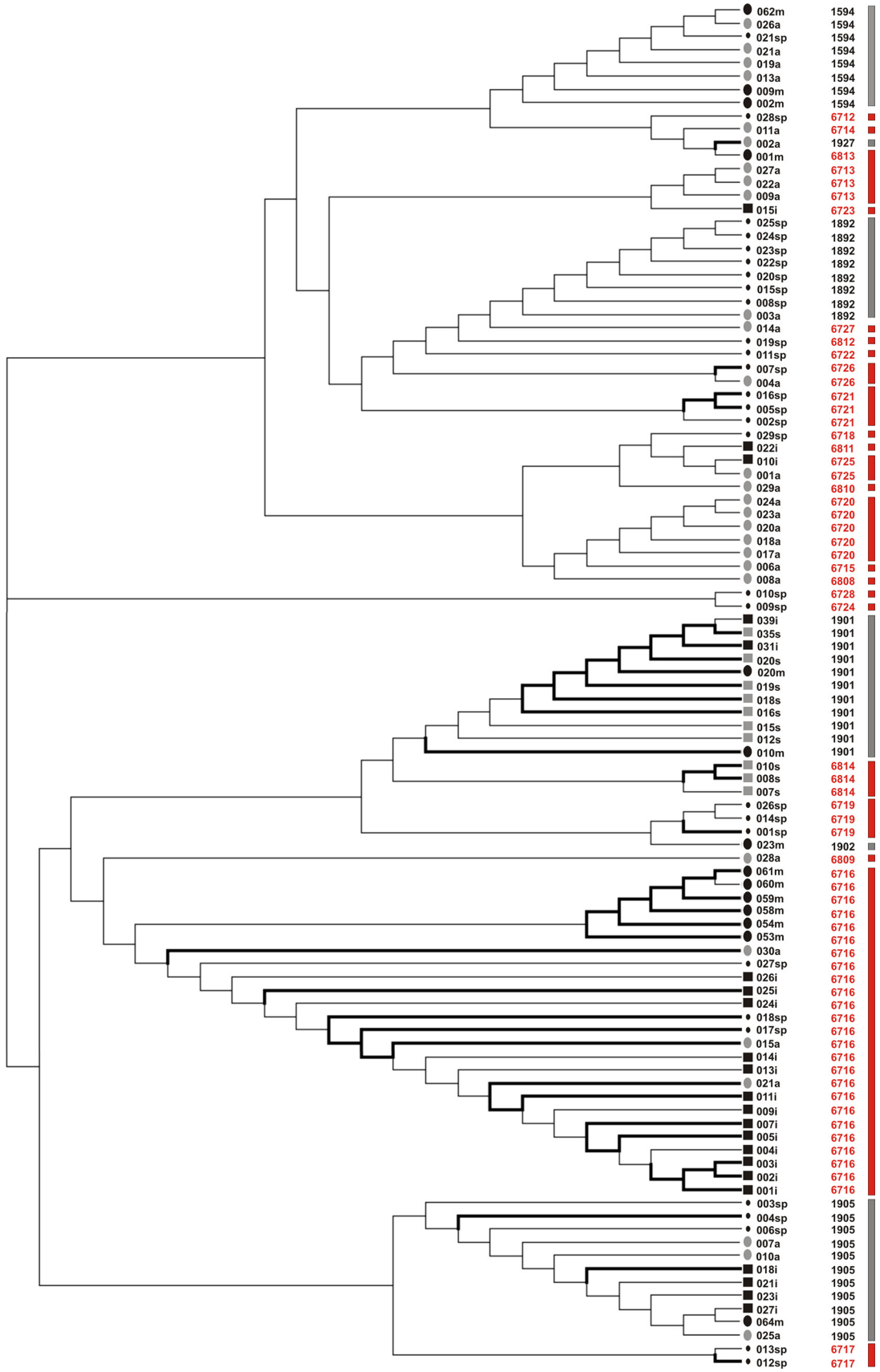



FIG. 3. Phylogenetic tree based on analysis of concatenated sequences of seven housekeeping gene fragments (MLST scheme). Newly described STs are indicated in red. Clinical *N. gonorrhoeae* strains ($n = 103$) are named according to Table S2 in the supplemental material. Isolate origins: Irkutsk ($n = 22$), black square; Samara ($n = 10$), gray square; Murmansk ($n = 14$), large black circle; St. Petersburg ($n = 29$), small black circle; Arkhangelsk ($n = 28$), large gray circle. Multiresistant isolates (i.e., isolates resistant to at least two antibiotics) are indicated by thick lines.

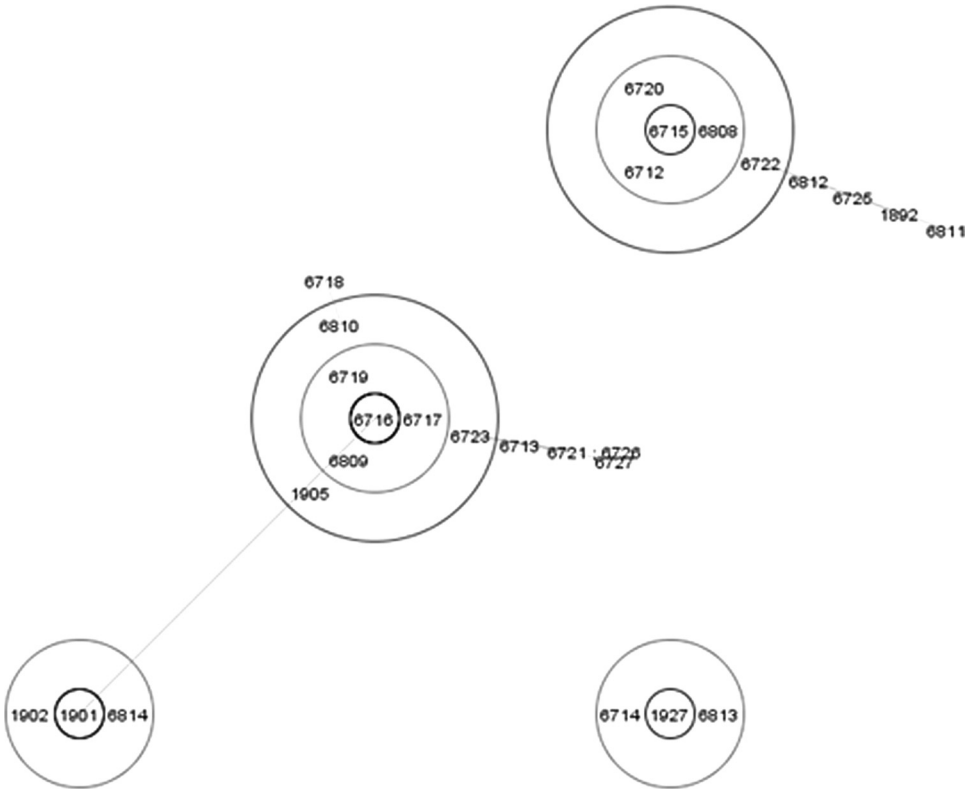



FIG. 4. Population snapshot of clinical *N. gonorrhoeae* isolates ($n = 103$) typed in the present study. The snapshot was created by the eBURST algorithm applied for the analysis of MLST data. The circles represent STs differing in only one housekeeping gene sequence from the founder genotype (in the center). Lines connect the other evolutionarily related STs, i.e., those sharing six of seven alleles. Each centered group was considered a clonal complex. ST1594 was identified as a singleton.

ing intermediate susceptibility to PEN, resistance to TET, and (especially) susceptibility to CIP.

## DISCUSSION

In recent years, many molecular approaches for *N. gonorrhoeae* typing have been suggested: *opa* typing (23, 25), *por* typing (7, 15, 34, 39), amplified fragment length polymorphism (24, 32), restriction fragment length polymorphism (3, 15), and some others, but no single typing scheme has been generally adopted, and the lack of such a standard typing method has impeded the sharing of epidemiological data between laboratories. The recently developed NG-MAST (19) and MLST (1, 38) approaches look the most promising in this field.

In our research, four different schemes of molecular typing have been adopted and used to study gonococcal strains circulating in the Russian Federation. The traditional serotyping procedure is based on an analysis of protein variability. The other typing schemes—*por* typing, NG-MAST, and MLST—are nucleic acid-based techniques.

Each tested technique typed all of the isolates, but their discriminatory powers were different. According to our data, the discriminatory power of the traditional serotyping approach was 0.82 and did not satisfy the value of ≥0.90 desirable for effective typing systems (9). Although this disadvantage of serotyping was discovered 10 years ago (37), this method can still be useful when a comparison of modern and old data is required.

The discriminatory power of each of the nucleic acid-based

TABLE 2. Allele frequencies within *N. gonorrhoeae* isolates collected in the Russian Federation and the United Kingdom[a]

| Isolate type[b] (no. of isolates) | No. of unique variants | | | | | | |
|---|---|---|---|---|---|---|---|
| | *abcZ* | *adk* | *aroE* | *fumC* | *gdh* | *pdhC* | *pgm* |
| RUS (103) | **5** | 2 | 2 | **7** | **5** | 4 | 3 |
| UK (108) | **7** | 3 | 2 | **6** | **9** | 3 | 3 |

[a] The most variable alleles are indicated in boldface.
[b] RUS, isolates collected in the Russian Federation; UK, isolates collected in the United Kingdom.

TABLE 3. Multilocus linkage disequilibrium analysis of *N. gonorrhoeae* isolates collected in the Russian Federation and the United Kingdom[a]

| Isolate type[b] (no. of isolates) | Ve | Vo | $I_A$ | *P* | $I^S_A$ |
|---|---|---|---|---|---|
| RUS (103) | 1.5081 | 3.0065 | 0.9935 | 0.000 | 0.1656 |
| UK (108) | 1.4803 | 1.3165 | 0.1244 | 0.008 | 0.0207 |

[a] Values for the expected variance (Ve), the observed variance (Vo), and the index of association ($I_A$), with the associated *P* value and the standardized index of association ($I^S_A$), are given.
[b] RUS, isolates collected in the Russian Federation; UK, isolates collected in the United Kingdom.

TABLE 4. Distribution of 103 *N. gonorrhoeae* isolates by susceptibility level against different sequence types according to MLST

| ST according to MLST | No. of isolates (% total) | Most prevalent phenotype (% isolates with the indicated phenotype)[a] | | | *P* |
|---|---|---|---|---|---|
| | | PEN | TET | CIP | |
| ST1594 | 8 (7.8) | S/I | S (87.5) | S (100.0) | 0.0573 |
| ST1892 | 8 (7.8) | S (87.5) | S (87.5) | S (100.0) | 0.0079 |
| **ST1901** | **11 (10.7)** | **I (100.0)** | **R (90.1)** | **R (100.0)** | **0.0002** |
| ST1905 | 11 (10.7) | I (81.8) | R (81.8) | S (100.0) | 0.0032 |
| **ST6716** | **25 (24.3)** | **I (84.0)** | **R (65.0)** | **R (100.0)** | **0.0000** |
| ST6720 | 5 (4.8) | S/I | S (100.0) | S (100.0) | 0.1525 |

[a] S, susceptible; I, intermediate resistant; R, resistant (in accordance with the Clinical and Laboratory Standards Institute recommendations for penicillin [PEN], tetracycline [TET], and ciprofloxacin [CIP]). STs strongly associated with multidrug resistance are indicated in boldface. S/I, Almost equal numbers of isolates with each phenotypes.

techniques was quite high. Both *por* typing and NG-MAST satisfied the value of ≥0.95 recommended for new typing methods by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (36), in contrast to the MLST scheme.

Generally, the *N. gonorrhoeae* typing data obtained by *por* typing and NG-MAST appeared to be very similar. Both methods divided gonococcal strains into two large nonoverlapping groups in accordance with *porB* alleles (or serovars in terms of proteins). Both methods had comparable discriminatory power (0.97 and 0.98 for *por* typing and NG-MAST, respectively) sufficient for identifying transmission clusters of *N. gonorrhoeae*. In addition, there was no clear geographic clustering of specific *por* types or STs according to NG-MAST found within the examined group.

As far as *por* typing is concerned, different trends in the evolution of *porB* alleles were found. Earlier, such a phenomenon was described by Smith et al. in 1995 (28) and Posada et al. in 2000 (31). This fact can be explained by different epidemiologies in the P1A and P1B homology groups. It was shown that blood isolates obtained during disseminated gonococcal infection generally belong to the P1A serovar (5, 29), whereas isolates from mucosal surfaces more often belong to the P1B serovar (21). It may be that the invasive process requires certain antigens exposed on the microbial cell surface that elucidate the influence of purifying selection on PIA gonococci.

In contrast to *por* typing, NG-MAST allows international comparisons of genotyping data. Unfortunately, most of the STs identified in our research were newly described STs. Of 23 known STs, ST1527, ST1534, ST1544, and ST1548 had previously been detected in *N. gonorrhoeae* samples obtained in Arkhangelsk (35). It should be noted that the most common ST in a European population, ST225 (26), was found in one isolate from Irkutsk.

Although the discriminatory power of MLST was slightly lower than that of *por* typing and NG-MAST, the obtained genotyping data looked very promising. First, the tested gonococci were divided into 30 different clusters aside from their serotypes. In spite of the fact that *N. gonorrhoeae* has earlier been proposed to have a nonclonal population structure (22, 30), four clonal complexes were identified in the analyzed group.

Moreover, using data from public MLST site (http://pubmlst.org/neisseria) allowed us to compare the *N. gonorrhoeae* populations in the Russian Federation and the United Kingdom. The similar variabilities of alleles in both populations reflect the universality of this typing scheme and its applicability for worldwide studies.

Despite the significant linkage disequilibrium detected for both groups, the *N. gonorrhoeae* population in Russia seems more homogeneous, more clonal than in the United Kingdom. For Russian isolates, the observed variance was much greater than the maximum variance obtained in 1,000 trials ($P = 0.000$). In combination with the $I_A$ and $I^S_A$ values, this suggests that the population structure of the examined *N. gonorrhoeae* isolates is highly clonal. It was confirmed by the eBURST analysis data shown that the majority of gonococci can be combined into four clonal complexes with only a singleton formed by ST1594.

Only 6 STs discovered here by MLST were described elsewhere, and 24 STs were newly elaborated. It should be mentioned that ST1905 was previously identified only in gonococci isolated in Uzbekistan and Russia (1). In accordance with MLST findings, there was no evidence for geographical structuring among the isolates examined, and a strong relationship between the susceptibility profile of *N. gonorrhoeae* and certain STs was discovered. Moreover, two STs clearly associated with multidrug resistance were determined by eBURST to be evolutionarily related (Fig. 4). If we take into account the geographic heterogeneity of each cluster (Fig. 4; see also Table S2 in the supplemental material), it seems that the multiresistant strains in Russia spread as two large clonal groups corresponding to MLST ST6716 and ST1901.

In conclusion, we assert that the methods based on nucleic acid variability are more suitable for *N. gonorrhoeae* molecular typing than the ones based on changes in proteins. Among these, the NG-MAST displayed the highest resolution that is useful in monitoring short-term transmission patterns of the organism and is more appropriate for local outbreak investigations. Meanwhile, MLST, in spite of its lower discriminating potential, seems to be the best choice for global epidemiological studies.

## ACKNOWLEDGMENTS

This study was supported by the Russian Ministry of Health (development contract 06/311 [30 May 2008]).

We thank V. A. Karpov for oligonucleotide primer synthesis and M. M. Chukin and T. A. Akopian for assistance with the sequence instrumentation.

We used the NG-MAST database (http://www.ng-mast.net), Department of Infectious Disease Epidemiology, Imperial College London,

and the *Neisseria* MLST website (http://pubmlst.org/neisseria/) developed by Keith Jolley and Man-Suen Chan (13) and located at the University of Oxford.

## REFERENCES


1. **Bennett, J. S., K. A. Jolley, P. F. Sparling, N. J. Saunders, C. A. Hart, I. M. Feavers, and M. C. Maiden.** 2007. Species status of *Neisseria gonorrhoeae*: evolutionary and epidemiological inferences from MLST. BMC Biol. **5:**35.
2. **Bilek, N., I. M. Martin, G. Bell, G. R. Kinghorn, C. A. Ison, and B. G. Spratt.** 2007. Concordance between *Neisseria gonorrhoeae* genotypes recovered from known sexual contacts. J. Clin. Microbiol. **45:**3564–3567.
3. **Bindu, M. P., P. Rajendran, S. P. Thyagarajan, N. P. Pramod, U. A. Rao, M. Ganapathy, T. Kurien, and the STD Study Group.** 2002. Molecular typing of *Neisseria gonorrhoeae* from hospital and community isolates by restriction fragment length polymorphism. Int. J. STD AIDS **13:**124–130.
4. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. H. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
5. **Cannon, J. G., T. M. Buchanan, and P. F. Sparlingi.** 1983. Confirmation of association of protein I serotype of *Neisseria gonorrhoeae* with ability to cause disseminated infection. Infect. Immun. **40:**816–819.
6. **Francisco, A. P., M. Bugalho, M. Ramirez, and J. A. Carriço.** 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinform. **10:**152.
7. **Fudyk, T. C., I. W. Maclean, J. N. Simonsen, E. N. Njagi, J. Kimani, R. C. Brunham, and F. A. Plummer.** 1999. Genetic diversity and mosaicism at the *por* locus of *Neisseria gonorrhoeae*. J. Bacteriol. **181:**5591–5599.
8. **Hobbs, M. M., T. M. Alcorn, R. H. Davis, W. Fischer, J. C. Thomas, I. Martin, C. Ison, P. F. Sparling, and M. S. Cohen.** 1999. Molecular typing of *Neisseria gonorrhoeae* causing repeated infections: evolution of porin during passage within a community. J. Infect. Dis. **179:**371–381.
9. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
10. **Huson, D. H., D. C. Richter, C. Rausch, T. Dezulian, M. Franz, and R. Rupp.** 2007. Dendroscope: an interactive viewer for large phylogenetic trees. BMC Bioinform. **8:**460.
11. **Ilina, E. N., V. A. Vereshchagin, A. D. Borovskaya, M. V. Malakhova, S. V. Sidorenko, N. C. Al-Khafaji, A. A. Kubanova, and V. M. Govorun.** 2008. Relation between genetic markers of drug resistance and susceptibility profile of clinical *Neisseria gonorrhoeae* strains. Antimicrob. Agents Chemother. **52:**2175–2182.
12. **Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden.** 2001. Sequence type analysis and recombinational tests (START). Bioinformatics **17:**1230–1231.
13. **Jolley, K. A., M. S. Chan, and M. C. Maiden.** 2004. MlstdbNet: distributed multilocus sequence typing (MLST) databases. BMC Bioinform. **5:**86.
14. **Kubanova, A., N. Frigo, A. Kubanov, S. Sidorenko, T. Priputnevich, T. Vachnina, N. Al-Khafaji, S. Polevshikova, V. Solomka, M. Domeika, and M. Unemo.** 2008. National surveillance of antimicrobial susceptibility in *Neisseria gonorrhoeae* in 2005–2006 and recommendations of first-line antimicrobial drugs for gonorrhea treatment in Russia. Sex. Transm. Infect. **84:**285–289.
15. **Lau, Q. C., V. T. Chow, and C. L. Poh.** 1995. Differentiation of *Neisseria gonorrhoeae* strains by polymerase chain reaction and restriction fragment length polymorphism of outer membrane protein IB genes. Genitourin. Med. **71:**363–366.
16. **Ling, A. E., M. C. Bash, F. Lynn, N. A. Lister, P. Zhu, S. M. Garland, C. K. Fairley, and S. N. Tabrizi.** 2007. Evaluation of PorB variable region typing of *Neisseria gonorrhoeae* using PCR-ELISA in samples collected from men who have sex with men. J. Clin. Lab. Anal. **21:**237–243.
17. **Lynn, F., M. M. Hobbs, J. M. Zenilman, F. M. Behets, K. Van Damme, A. Rasamindrakotroka, and M. C. Bash.** 2005. Genetic typing of the porin protein of *Neisseria gonorrhoeae* from clinical noncultured samples for strain characterization and identification of mixed gonococcal infections. J. Clin. Microbiol. **43:**368–375.
18. **Martin, I. M. C., E. Foreman, V. Hall, A. Nesbitt, G. Forster, and C. A. Ison.** 2007. Non-cultural detection and molecular genotyping of *Neisseria gonorrhoeae* from a piece of clothing. J. Med. Microbiol. **56:**487–490.
19. **Martin, I. M. C., C. A. Ison, D. M. Aanensen, K. A. Fenton, and B. G. Spratt.** 2004. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J. Infect. Dis. **189:**1497–1505.
20. **Monfort, L., V. Caro, Z. Devaux, A.-S. Delannoy, S. Brisse, and P. Sednaoui.** 2009. First *Neisseria gonorrhoeae* genotyping analysis in France: identification of a strain cluster with reduced susceptibility to ceftriaxone. J. Clin. Microbiol. **47:**3540–3545.
21. **Morse, S. A., P. G. Lysko, L. McFarland, J. S. Knapp, E. Sandstrom, C. Critchlow, and K. K. Holmes.** 1982. Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. Infect. Immun. **37:**432–438.
22. **O'Rourke, M., and E. Stevens.** 1993. Genetic structure of *Neisseria gonorrhoeae* populations: a non-clonal pathogen. J. Gen. Microbiol. **139:**2601–2603.
23. **O'Rourke, M., C. A. Ison, A. M. Renton, and B. G. Spratt.** 1995. Opa-typing: a high-resolution tool for studying the epidemiology of gonorrhea. Mol. Microbiol. **17:**865–875.
24. **Palmer, H. M., and C. Arnold.** 2001. Genotyping *Neisseria gonorrhoeae* using fluorescent amplified fragment length polymorphism analysis. J. Clin. Microbiol. **39:**2325–2329.
25. **Palmer, H. M., J. P. Leeming, and A. Turner.** 2001. Investigation of an outbreak of ciprofloxacin-resistant *Neisseria gonorrhoeae* using a simplified opa-typing method. Epidemiol. Infect. **126:**219–224.
26. **Palmer, H. M., H. Young, C. Graham, and J. Dave.** 2008. Prediction of antibiotic resistance using *Neisseria gonorrhoeae* multi-antigen sequence typing. Sex. Transm. Infect. **84:**280–284.
27. **Poh, C. L., Q. C. Lau, and V. T. Chow.** 1995. Differentiation of *Neisseria gonorrhoeae* IB-3 and IB-7 serovars by direct sequencing of protein IB gene and pulsed-field gel electrophoresis. J. Med. Microbiol. **43:**201–207.
28. **Posada, G. D., K. A. Crandall, M. Nguyen, J. C. Demma, and R. P. Viscidi.** 2000. Population genetics of the *porB* gene of *Neisseria gonorrhoeae*: different dynamics in different homology. Mol. Biol. Evol. **17:**423–436.
29. **Sandstrom, E. G., J. S. Knapp, L. B. Reller, S. E. Thompson, E. W. I. Hook, and K. K. Holmes.** 1984. Serogrouping of *Neisseria gonorrhoeae*: correlation of serogroup with disseminated gonococcal infection. Sex. Transm. Dis. **11:**77–80.
30. **Smith, J. M., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. U. S. A. **90:**4384–4388.
31. **Smith, N. H., J. M. Smith, and B. G. Spratt.** 1995. Sequence evolution of the *porB* gene of *Neisseria gonorrhoeae* and *Neisseria meningitidis*: evidence of positive Darwinian selection. Mol. Biol. Evol. **12:**363–370.
32. **Spaargaren, J., J. Stoof, H. Fennema, R. Coutinho, and P. Savelkoul.** 2001. Amplified fragment length polymorphism fingerprinting for identification of a core group of *Neisseria gonorrhoeae* transmitters in the population attending a clinic for treatment of sexually transmitted diseases in Amsterdam, The Netherlands. J. Clin. Microbiol. **39:**2335–2337.
33. **Tapsall, J. W., E. A. Limnios, and D. Murphy on behalf of the Australian Gonococcal Surveillance Programme.** 2008. Analysis of trends in antimicrobial resistance in *Neisseria gonorrhoeae* isolated in Australia, 1997–2006. J. Antimicrob. Chemother. **61:**150–155.
34. **Thompson, D. K., C. D. Deal, C. A. Ison, J. M. Zenilman, and M. C. Bash.** 2000. A typing system for *Neisseria gonorrhoeae* based on biotinylated oligonucleotide probes to PIB gene variable regions. J. Infect. Dis. **181:**1652–1660.
35. **Unemo, M., V. Vorobieva, N. Firsova, T. Ababkova, I. Leniv, B. C. Haldorsen, H. Fredlund, and V. Skogen.** 2007. *Neisseria gonorrhoeae* population in Arkhangelsk, Russia: phenotypic and genotypic heterogeneity. Clin. Microbiol. Infect. **13:**873–878.
36. **van Belkum, A., P. T. Tassios, L. Dijkshoorn, S. Haeggman, B. Cookson, N. K. Fry, V. Fussing, J. Green, E. Feil, P. Gerner-Smidt, S. Brisse, M. Struelens, and The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group on Epidemiological Markers (ESGEM).** 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin. Microbiol. Infect. **2007**(Suppl. 3)**:**1–46.
37. **van Looveren, M., C. A. Ison, M. Ieven, P. Vandamme, I. M. Martin, K. Vermeulen, A. Renton, and H. Goossens.** 1999. Evaluation of the discriminatory power of typing methods for *Neisseria gonorrhoeae*. J. Clin. Microbiol. **37:**2183–2188.
38. **Viscidi, R. P., and J. C. Demma.** 2003. Genetic diversity of *Neisseria gonorrhoeae* housekeeping genes. J. Clin. Microbiol. **41:**197–204.
39. **Viscidi, R. P., J. C. Demma, J. Gu, and J. Zenilman.** 2000. Comparison of sequencing of the *por* gene and typing of the *opa* gene for discrimination of *Neisseria gonorrhoeae* strains from sexual contacts. J. Clin. Microbiol. **38:** 4430–4438.
40. **Whiley, D. M., N. Goire, E. S. Ray, A. Limnios, S. B. Lambert, M. D. Nissen, T. P. Sloots, and J. W. Tapsall.** 2010. *Neisseria gonorrhoeae* multi-antigen sequence typing using non-cultured clinical specimens. Sex. Transm. Infect. **86:**51–55.