Molecular surveillance of clinical Neisseria gonorrhoeae isolates in Russia.


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Running title: N. gonorrhoeae molecular typing

ABSTRACT

The choice of adequate methods for epidemiological purpose remains a challenging problem of Neisseria gonorrhoeae molecular monitoring. In this study collection of geographically unrelated gonococci (n = 103) isolated in Russian clinics was comparably tested by traditional serotyping scheme, por-typing, Neisseria gonorrhoeae Multi Antigen Sequence Typing (NG-MAST) and Multilocus Sequence Typing (MLST). It was shown, that according to sequencing data a third of strains carried new porB1 alleles as well as tbpB ones, and more than half samples had new sequence types by NG-MAST or by MLST. The discriminatory power for each typing method was calculated using the Hunter–Gaston discriminatory index (HGDI, D). Commonly, the modern nucleic acid based typing methods (por-typing, NG-MAST, MLST) appeared more efficient than the classical serotyping scheme. While the traditional serotyping gave the D = 0.82, the por-typing, NG-MAST, and
MLST allowed to reach $D = 0.97$, $0.98$, and $0.91$, respectively. Each typing technique revealed the distribution of gonococci slightly correlated with their geographical sources. But only the MLST sequence types were highly associated with certain phenotypes. While the ST1594, ST1892 and ST6720 were typical for susceptible gonococci, the ST1901 and ST6716 were undoubtedly associated with multidrug resistant phenotype. We can conclude that every tested nucleic acid based typing method is suitable for \textit{N. gonorrhoeae} molecular surveillance. However the MLST seems to answer large-scale epidemiological purposes while the NG-MAST as well as \textit{por}-typing is more appropriated for local outbreak investigation.

\section*{INTRODUCTION}

The choice of adequate epidemiological approach for \textit{Neisseria gonorrhoeae} typing remains a topical problem of public health control strategies. The worldwide spreading of drug resistant strains requires the typing methods to be introduced into a national surveillance programs, that is already realized within the Australian Gonococcal Surveillance Programme (AGSP) [33]. In Russia the multiresistant strains which are resistant to penicillin, tetracycline and fluoroquinolones are prevalent and reach up to 60 % in some regions [14]. As is generally known the majority of resistance mechanisms in \textit{N. gonorrhoeae} are linked to the mutations in genomic DNA and a wide spreading of such mutations was shown in the Russian population of gonorrhea [11]. In fact, the chromosomally mediated drug resistance expands clonally in bacterial population. So, tracing the spreading of gonorrhea by any kind of strain differentiation methods is of an urgent importance. On the other hand, population genetics is important in understanding the evolutionary history, epidemiology, and population dynamics of pathogens.

Nowadays widespread adoption of the molecular diagnosis of gonorrhea could compromise traditional bacteriological cultivation in routine practice and highlight the needs in the development of molecular tools for \textit{N. gonorrhoeae} typing. There are several promising
approaches existing in this field. One of them is por-typing [27, 8, 7] based on comparative analysis of porB gene nucleotide sequences. It possesses rather high discriminatory power [39] and presents data in format comparable with serotyping data. It should be mentioned that PorB1 protein plays a key role in 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 serotype determined in accordance with antigenic properties of PorB1 proteins [7, 29, 21].

Today, the NG-MAST (N. gonorrhoeae multiantigen sequence typing) [19] claims 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 coding superficial antigens of gonococcus and being therefore under the positive selection [19]. Online accessible data base (www.ng-mast.net) collects current information on the sequence types of gonococci isolates in different regions of the world, and it can be an efficient instrument for objective estimation of genetic variability of microbial population and tracing the spreading of infection [2]. Moreover, the association of NG–MAST sequence type with antibiotic resistance profile was shown by some investigators [26, 20].

In addition, NG–MAST, which is usually carried out on DNA extracted from pure bacterial culture, can be performed directly from non-cultured samples such as a piece of clothing [18] or some of clinical specimens [40]. Though some authors reported a successful application of NG-MAST to urogenital specimens (urine, swabs from cervix, urethra, vagina) and to rectal swabs, this typing method was found less suitable for throat swabs, due to cross-reaction with commensal Neisseria species. It seems that the direct typing schemes utilized probe hybridization methods [16, 17] for a broad spectrum of clinical samples will be more convenient than sequencing.

The opposite way – analysis of conservative, presumably selective neutral housekeeping genes is realized by MLST (multilocus sequence typing) [38, 1]. Having a
sufficient number of allelic variants and being characterized by slow accumulation of
mutations, these genes reflect the natural evolution of microbial population and common
trends in gonorrhea spreading.

Commonly the effectiveness of *N. gonorrhoeae* surveillance depends on methods used
for species identification and typing of clinical isolates. However, taking into account the
variety of typing systems and absence of world experience in their application to monitoring
of gonococci, the question about a unique objective method of revealing the relation between
clinical isolates remains opened. The goal of this study was to evaluate a number of
approaches including *por*-typing, NG-MAST, MLST and a serotyping for typing
differentially unrelated gonococci isolates in Russia.

This work was partially presented during the 16th International Pathogenic Neisseria

**METHODS**

**Bacterial isolates.** *N. gonorrhoeae* clinical isolates collected in different regions of
Russia during the years 2004 – 2005 were obtained from Central Research Institute of
Dermatology and Venereology. All isolates were identified as *N. gonorrhoeae* and tested for
serotype and for susceptibility to penicillin G, tetracycline, ciprofloxacin, spectinomycine 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 a
temperature of – 20 °C.

Amplification reactions were carried out in 10 µl of 66 mM Tris-HCl pH 9.0; 16.6 mM
(NH₄)₂SO₄; 2.5 mM MgCl₂; 0.2 mM of each dNTP; 5 pmol of each primers (see Table S1 in
the supplementary material) and 1 unit of TaqPolymerase (Fermentas, Lithuania) under
following conditions: 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 15 s, in 35 cycles. A programmed thermocycler TETRAD DNA ENGINE (MJ Research Inc.) has been used.

Dephosphorylation of the 5’-end phosphate groups of dNTPs and cleavage of primers in the post-amplification 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 the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, USA; Hitachi, Japan) according to the manufacturer’s instruction.

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

*N. gonorrhoeae* multi-antigen or multilocus sequence typing.

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

**Data analysis.** Sequences were aligned using ClustalX 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 nucleotide Tajima-Nei model). Further classification was constructed using Neighbour-Joining method basing on bootstrap tree stability test (500 iterations).
Visual mapping of multidrug resistance and place of strain isolation were carried out 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 single numerical index of discrimination (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, \(S\) - the number of different types, and \(n_j\) - the number of strains belonging to the \(j^{th}\) type.

The calculated data was interpreted with commonly accepted decision that a \(D\) of greater than 0.90 is desirable for confident typing method [9].

A recombination test, the index of association (\(I_A\) and \(I_S\)) was performed using the START2 program [12]. Allele profile data were analyzed in eBURSTv3 version (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 the significance level at least \(p<0.05\). Statistical analysis was performed with STATISTICA 7.0 software.

**RESULTS**

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

Protein based typing method
Traditional serotyping procedure divided all typing isolates into 11 different groups with the prevalence of 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. 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 PIA6 serotype was found in 10 (9.7 %) of isolates, and one sample belonged to PIA10 serotype. The calculated D was found 0.82 which testifies that discriminatory power of serotyping is insufficient to prove an effective typing method.

Nucleic acid based typing methods

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

por-typing. Alignment of 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 cut-off value of 0.1 (genetic distance) was used 58 clusters could be delineated. Expectedly all gonococci were separated into two large groups corresponding to porB1a (n = 11) and porB1b (n = 92) allele of porB gene. Comparative analysis of nucleotide polymorphism showed a much higher variability for porB1b allele than for porB1a. Variability for porB1b allele became apparent in a number of exposed allelic variants and polymorphic sites within each group. Moreover, three, six or twelve nucleotides insertions as well as six nucleotides deletions were found for several isolates possessing the porB1b allele. Analysis of non-synonymous/synonymous substitution rate ratio revealed the different trends in natural selection of porB alleles (Table 1). While the influence of positive diversifying selection was shown for porB1b allele, the porB1a allele seemed to be under the purifying selection. Commonly the discriminatory index for this typing method was shown 0.97 that is quite sufficient for epidemiological purposes.
Comparative analysis of internal fragments of porB genes (490 bp) via the public accessible database on the NG-MAST website (http://www.ng-mast.net) showed that a third of tested group carried new por alleles unrepresented in 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, por-90 cluster was clearly associated with PIA6 serotype, por-91, por-164 and por-232 clusters contained gonococci isolated especially in Irkutsk, Samara and Murmansk, respectively.

NG-MAST. Based on analysis of porB fragments as well as tbpB ones all isolates were characterized by NG-MAST. There were found 51 and 27 variants of porB and tbpB alleles, respectively, which resulted in the assignment of 61 different sequence types (STs). Phylogenetic tree based on the concatenated sequences of two loci is represented 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 PPNG collected in St.Petersburg (n = 3) and Murmansk (n = 1). Meanwhile about a half of the isolates (n = 50) belonged to the new 39 STs described for the first time. Generally, there was no clear geographic clustering of specific NG-MAST types found within the country. In some cases, certain ST was found for gonococci isolated in the same place, for example, ST205 in Samara, ST285 in Murmansk, and ST972 in Irkutsk. However, it is likely to reflect the local outbreaks rather than the global spread of infection. The calculated index of discrimination for this typing method amounted 0.98 that reflected the high discriminatory power of NG-MAST.

MLST. According to MLST data, the 30 different groups (STs) were discovered. Among them thirteen ST clusters of between two and 25 isolates were identified. ST6716 (n = 25) were found 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 seventeen STs were represented by single isolates. Concatenated sequences of seven housekeeping gene fragments
were analyzed by START 2.0 software. Phylogenetic tree constructed using Neighbour-Joining method is shown in Fig. 3. The calculated D was found 0.91 for MLST that means its applicability as an effective typing method.

Analysis by eBurst identified four non-overlapping 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 hyper-invasive genotypes. Among them ST1901 and ST6716 were evolutionary related, sharing six out of seven alleles.

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

**Genotyping data and antimicrobial resistance.**

The N. gonorrhoeae clinical isolates distribution according to different nucleic acid based typing schemes (por-typing, NG-MAST, MLST) was compared with their susceptible profile. As many of STs were represented by occasional isolates, only the clusters which contained five or more members have been considered. Among them the NG-MAST ST205 (n = 6), ST285 (n = 7) and ST282 (n = 5) were non-ambiguously associated with multidrug resistance, i.e., intermediate susceptibility or resistance to penicillin, tetracycline and ciprofloxacin. The other NG MAST ST1043 (n = 8) included gonococci non-susceptible to penicillin, resistant to tetracycline and susceptible to ciprofloxacin. Appropriately, the same isolates formed correspondent clusters in accordance with por-typing, too.

The evident relation between N. gonorrhoeae phenotype and certain ST by MLST was observed (Table 4). While the ST1594, ST1892 and ST6720 were typical for susceptible gonoccci, the ST1901 and ST6716 were undoubtedly associated with multidrug resistance.
The ST1905 comprised isolates displaying the intermediate susceptibility to penicillin, resistance to tetracycline and especially susceptibility to ciprofloxacin.

**DISCUSSION**

Over recent years many molecular approaches for *N. gonorrhoeae* typing have been suggested: *opa* typing [23, 25], *por* typing [15, 39, 34, 7], AFLP [24, 32], RFLP [3, 15] and some other, but no single typing scheme has been generally adopted, and the lack of such kind of a typing method has impeded the sharing of epidemiological data between laboratories. The recently developed NG-MAST [19] and MLST [38, 1] schemes look the most perspective in this field.

In our research, four different schemes of molecular typing have been adopted and applied for studying the gonococci circulating in Russian Federation. The traditional serotyping procedure was based on analysis of protein variability. The other typing schemes – *por*-typing, NG-MAST and MLST were nucleic acid based techniques.

Each tested technique typed all isolates, but their discriminatory power was different. According to our data, the discriminatory power of 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 ten years ago [37], this method can still be useful when a comparison of the modern and old data is required.

Discriminatory power of each of the nucleic acid based 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, *N. gonorrhoeae* typing data obtained by *por*-typing and NG-MAST appeared to be very similar. Both methods divided gonococci into two large non-overlapping 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 the different trends in evolution of *porB* alleles was found. Earlier such phenomenon has just been described by Smith et al. in 1995, and Posada et al. in 2000 [31, 28]. This fact can be explained by a different epidemiology of P1A and P1B homology groups. It was shown that blood isolates during disseminated gonococcal infection belong especially to the P1A serovar [29, 5], whereas isolates from mucosal surfaces more often belong to the P1B serovar [21]. One can suggest that the invasive process requires the certain antigens exposed on microbial cell surface that elucidates the influence of purifying selection on PIA gonococci.

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

Although the discriminatory power of MLST was slightly lower than 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 this fact that *N. gonorrhoeae* has earlier been proposed to have a nonclonal population structure [30, 22], the four clonal complexes were identified in analyzed group.

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

Despite the significant linkage disequilibrium was detected for both groups, *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 1000 trial (p = 0.000). In combination with the $I_A$ and $I^S_A$ values this would suggest that the population structure of the examined *N. gonorrhoeae* is highly clonal.

It was confirmed by eBurst analysis data shown that the majority of gonococci can be combined into four clonal complexes with only singleton formed by ST1594.

Only six STs discovered in this study by MLST were formerly described, and the 24 ones were newly elaborated. It should be mentioned that the known ST1905 was previously identified only in gonococci isolated in Uzbekistan and Russia [1]. While in accordance with MLST there was no evidence for geographical structuring among the isolates examined, the strong relation between susceptible profile of *N. gonorrhoeae* and certain ST was discovered. Moreover, two STs clearly associated with multidrug resistance were evolutionary related by eBurst (Fig. 5). Taking in account the geographic heterogeneity of each cluster (Fig. 4; see also Table S2 in the supplementary material) it seems that the multiresistant strains in Russia spread as two large clonal groups corresponded to MLST ST6716 and ST1901.

In conclusion, we can 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 appropriated for local outbreaks investigation. Meanwhile MLST in spite of its lower discriminating potential seems to be the best choice for global epidemiology purpose.

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This publication made use of Neisseria Gonorrhoeae Multi Antigen Sequence Typing databases and website (http://www.ng-mast.net) hosted at the Department of Infectious Disease Epidemiology, Imperial College London, and of the Neisseria Multi Locus Sequence Typing website (http://pubmlst.org/neisseria/) developed by Keith Jolley and Man-Suen Chan [13] and sited at the University of Oxford.

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European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study


Table 1. Variability of entire $porB$ gene nucleotide sequences for studied *N. gonorrhoeae* clinical isolates.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Length, bp.</th>
<th>N</th>
<th>Na</th>
<th>Nps</th>
<th>$\pi$</th>
<th>$z$-test: $d_n &lt; d_s$</th>
<th>$z$-test: $d_n = d_s$</th>
<th>$z$-test: $d_n &gt; d_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$porB$</td>
<td>933 - 1020</td>
<td>103</td>
<td>58</td>
<td>257</td>
<td>0.062</td>
<td>0.0013</td>
<td>0.0032</td>
<td>1.0</td>
</tr>
<tr>
<td>$porB_{1a}$</td>
<td>933</td>
<td>11</td>
<td>2</td>
<td>18</td>
<td>0.004</td>
<td>1.0</td>
<td>0.0076</td>
<td>0.0038</td>
</tr>
<tr>
<td>$porB_{1b}$</td>
<td>990 - 1020</td>
<td>92</td>
<td>56</td>
<td>135</td>
<td>0.030</td>
<td>0.21</td>
<td>0.44</td>
<td>1.0</td>
</tr>
</tbody>
</table>

- N – number of isolates;
- $Na$ – number of alleles;
- Nps – number of polymorphic sites;
- $\pi$ - value of nucleotide variability;
- $Z$-test was done for checking the null hypothesis whether the certain $porB$ allele is under the purifying selection ($d_n < d_s$), diversifying positive selection ($d_n > d_s$) or neutral selection ($d_n = d_s$).
- $d_n$ – number of nonsynonymous substitution
- $d_s$ – number of synonymous substitution.
Table 2. Allele frequencies within *N. gonorrhoeae* isolates collected in Russia Federation (RUS) and in the United Kingdom of Great Britain and Northern Ireland (UK). The most variable alleles are highlighted grey.

<table>
<thead>
<tr>
<th>Allele</th>
<th>abcZ</th>
<th>adk</th>
<th>aroE</th>
<th>fumC</th>
<th>gdh</th>
<th>pdhC</th>
<th>pgm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUS (n = 103)</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>UK (n = 108)</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3. Multilocus linkage disequilibrium analysis of the *N. gonorrhoeae* isolates collected in Russia Federation (RUS) and in the United Kingdom of Great Britain and Northern Ireland (UK). Values for expected variance (Ve), observed variance (Vo), index of association (Iₐ) with associated *P* value and standardized index of association (Iₐₛ) are given.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ve</th>
<th>Vo</th>
<th>Iₐ</th>
<th>P</th>
<th>Iₐₛ</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUS (103)</td>
<td>1.5081</td>
<td>3.0065</td>
<td>0.9935</td>
<td>0.000</td>
<td>0.1656</td>
</tr>
<tr>
<td>UK (108)</td>
<td>1.4803</td>
<td>1.3165</td>
<td>0.1244</td>
<td>0.008</td>
<td>0.0207</td>
</tr>
</tbody>
</table>
Table 4. Distribution of *N. gonorrhoeae* isolates (n = 103) by susceptibility level against different sequence types according to MLST. S – susceptible strains, I – intermediate resistant strains, R – resistant strains in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendation for penicillin (PEN), tetracycline (TET) and ciprofloxacin (CIP). STs strongly associated with multidrug resistance are highlighted grey.

<table>
<thead>
<tr>
<th>ST according to MLST</th>
<th>N (%)</th>
<th>Prevalent phenotype of susceptibility (per cent of isolates with certain phenotype, %)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEN</td>
<td>TET</td>
</tr>
<tr>
<td>1594</td>
<td>8 (7.8)</td>
<td>*S/I</td>
<td>S (87.5)</td>
</tr>
<tr>
<td>1892</td>
<td>8 (7.8)</td>
<td>S (87.5)</td>
<td>S (87.5)</td>
</tr>
<tr>
<td>1901</td>
<td>11 (10.7)</td>
<td>I</td>
<td>R (90.1)</td>
</tr>
<tr>
<td>1905</td>
<td>11 (10.7)</td>
<td>I (81.8)</td>
<td>R (81.8)</td>
</tr>
<tr>
<td>6716</td>
<td>25 (24.3)</td>
<td>I (84.0)</td>
<td>R (65.0)</td>
</tr>
<tr>
<td>6720</td>
<td>5 (4.8)</td>
<td>S/I</td>
<td>S (100.0)</td>
</tr>
</tbody>
</table>

* - almost equal isolates with different phenotypes.
Figure 1. Phylogenetic tree based on the analysis of 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. Isolates from Irkutsk (n = 22) are indicated by black square, from Samara (n = 10) – grey square, from Murmansk (n = 14) – large black circle, from St.Petersburg (n = 29) – little black circle, Arkhangelsk (n = 28) – large grey circle. The multiresistant (resistant at least to two antibiotics) isolates are shown by thick lines.
Figure 2. Phylogenetic tree based on the analysis of concatenated sequences of *porB1* and *tbpB* gene fragments (NG-MAST scheme). Newly described STs are selected by red; serovars P1A and P1B are indicated too. Clinical *N. gonorrhoeae* strains (n=103) are named according to Table S2. Isolates from Irkutsk (n = 22) are indicated by black square, from Samara (n = 10) – grey square, from Murmansk (n = 14) – large black circle, from St.Petersburg (n = 29) – little black circle, Arkhangelsk (n = 28) – large grey circle. The multi-resistant (resistant at least to two antibiotics) isolates are shown by thick lines.
Figure 3. Phylogenetic tree based on the analysis of concatenated sequences of seven housekeeping gene fragments (MLST scheme). Newly described STs are selected by red. Clinical *N. gonorrhoeae* strains (n=103) are named according to Table S2. Isolates from Irkutsk (n = 22) are indicated by black square, from Samara (n = 10) – grey square, from Murmansk (n = 14) – large black circle, from St.Petersburg (n = 29) – little black circle, Arkhangelsk (n = 28) – large grey circle. The multiresistant (resistant at least to two antibiotics) isolates are shown by thick lines.
Figure 4. Population snapshot of clinical *N. gonorrhoeae* isolates (n = 103) typing under the current investigation. The snapshot was created by the eBURST algorithm applied for the analysis of MLST data. The circles represented STs differing only in one housekeeping gene sequence from the founder genotype (in centre). Lines connected the other evolutionary related STs, sharing six out of seven alleles. Each centered group was considered as clonal complex. The ST1594 was identified as a singleton.