The emergence of antibiotic resistant strains poses a great concern for gonorrhea treatment. The aim of this study was to characterize penicillinase–producing Neisseria gonorrhoeae (PPNG) isolates collected in Italy in two time frames, 2003–2004 and 2014–2015. A total of 80 PPNG were characterized for the blaTEM gene variant and the plasmid type. Furthermore, gonococci were typed using Neisseria gonorrhoeae multiantigen sequence typing. Antibiotic susceptibility assay was performed for penicillin, ciprofloxacin, ceftriaxone, and spectinomycin by Etest and minimum inhibitory concentration (MIC) test strip methods. The β-lactamase production was detected using nitrocefin test. Among PPNG isolates, four blaTEM alleles were identified as follows: blaTEM-1, blaTEM-228, blaTEM-P14S, and blaTEM-135. The African plasmid possessed the blaTEM-1, blaTEM-228, and blaTEM-P14S, whereas blaTEM-135 was identified in Toronto/Rio and Asian plasmids. The percentage of isolates with the blaTEM-1-carrying African plasmid increased from 42.5% in 2003–2004 to 55% in 2014–2015; conversely, the isolates with blaTEM-135-carrying Toronto/Rio plasmid decreased from 57.5% to 35%. Among the isolates carrying the Toronto/Rio plasmids possessing blaTEM-135, sequence type (ST)661 and ST5624 were found to be the predominant STs in both periods 2003–2004 and 2014–2015, respectively. More than half of the PPNG isolates were resistant to ciprofloxacin. Increase in the isolates carrying the African plasmid possessing blaTEM-1 and a parallel decrease of the blaTEM-135-carrying Toronto/Rio plasmid was observed. Moreover, PPNG isolate harbored Toronto/Rio plasmid with blaTEM-135 belonged mainly to two major STs (ST661 and ST5624). Given the possible role of a mutated blaTEM gene as an additional mechanism to extended spectrum β-lactamase resistance, it is crucial to monitor gonococci carrying these resistance genes.

**Keywords:** antimicrobial resistance, blaTEM gene, penicillinase–producing Neisseria gonorrhoeae (PPNG), plasmid types

**Introduction**

Neisseria gonorrhoeae infection represents an important public health threat due to widespread multiple antimicrobial resistance. Over time, N. gonorrhoeae has developed resistance to a wide range of antibiotics and, more recently, to third-generation cephalosporins. For this reason, public health national and international initiatives aim to monitor the spread and the main molecular characteristics of resistant gonococci. Due to its attitude to alter genetic material, N. gonorrhoeae has developed several mechanisms of resistance through gene transfer or specific mutations, which result in the increase in the number of resistant gonococci. The worldwide dissemination of antimicrobial resistant (AMR) N. gonorrhoeae and the resistance plasmids carrying β-lactamase–encoding gene (blaTEM) has caused an increased interest in penicillinase–producing Neisseria gonorrhoeae (PPNG) isolates. PPNG results in high-level resistance to penicillin and, although no longer used for...
gonorrhea treatment, a significant proportion of strains have retained their resistance to this antibiotic.1,5

Several studies have described the prevalence and the molecular characterization of PPNG and the blaTEM gene variants.2–11

Among the eight plasmid types carrying the blaTEM gene, Asian, African, and Toronto/Rio plasmids were the most frequently described and widespread globally.1,5 The blaTEM gene encodes for β-lactamase able to hydrolyze the cyclic amide bond in the β-lactam ring inactivating the molecular activity.1–12

The blaTEM-1 and blaTEM-135, which only differ by one single nucleotide polymorphism, were the most commonly identified gene variants among PPNG isolates.1,5,6 Recently, a new blaTEM-220 carried by Toronto/Rio plasmid was identified in PPNG isolated in Argentina.8,9

Molecular typing by Neisseria gonorrhoeae multiantigen sequence typing (NG-MAST) has improved the molecular epidemiology of gonococci13; furthermore, an association between specific sequence types (STs) and blaTEM alleles was observed.9,11

In Italy, the proportion of PPNG isolates decreased significantly from 77% in 2003 to 7% in 2012.14 In our prior studies no further information about blaTEM variants was available. This prompted us to perform a retrospective analysis of plasmid and blaTEM variants responsible for penicillin resistance in 80 gonococi collected in Italy in two time frames, 2003–2004 and 2014–2015. Molecular typing by NG-MAST was evaluated to identify the most representative ST. Finally, the minimum inhibitory concentration (MIC) determination for the susceptibility to penicillin, ceftriaxone, ciprofloxacin, and spectinomycin was also determined.

Materials and Methods

PPNG isolate collection

N. gonorrhoeae isolates were collected by 14 laboratories collaborating with the Department Infectious Diseases, Istituto Superiore di Sanità in Rome, as Reference Center for the antimicrobial susceptibility study on gonorrhea and as part of European Gonococcal Antimicrobial Surveillance Program (EURO-GASP).15

The isolates were initially determined to be PPNG on the basis of nitrocefin disc testing (Oxoid Ltd.) after growth on Thayer-Martin agar (Oxoid Ltd.) with 1% of IsoVitalex (Oxoid Ltd.) at 37°C in a 5% CO2 atmosphere. For each isolate, information on the type of clinical specimen and of patient was anonymously provided and recorded using Epi Info software (version 3.3.2). Eighty N. gonorrhoeae isolates resulted in penicillin resistant and producing β-lactamase; they were randomly selected from two time frames 2003–2004 and 2014–2015, 40 isolates for each period.

Ethical approval was not required since the isolates were collected and stored as part of routine clinical care by the laboratories participating in the study.

Molecular characterization of blaTEM gene, plasmid type, and NG-MAST

Plasmid DNA was extracted from a fresh subculture using a boiling extraction method.8 Plasmid type and blaTEM gene were identified as already described.16,17 Multiple-sequence alignments of nucleotide and amino acid sequence were performed using ClustalW (http://ebi.ac.uk/Tools/msa/clustalo/). The amino acid sequences of β-lactamase were compared to the sequences in the Lactamase Engineering Database (http://laced.uni-stuttgart.de/) and numbering according to the scheme proposed by Ambler et al.18

Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen), according to the manufacturer’s instructions. Sequencing of porB and tpbB was achieved using primers and amplification parameters, as previously described.11 The porB and tpbB alleles, as well as the STs, were assigned according to NG-MAST website (http://ng-mast.net).

Antimicrobial susceptibility testing

All isolates were examined to define the MIC for penicillin, ciprofloxacin, ceftriaxone, and spectinomycin. Penicillin susceptibility was evaluated using Etest method (bioMérieux, Sweden) on isolate collected before 2009. Ciprofloxacin, ceftriaxone, and spectinomycin were all tested using MIC test strip method (Liofilchem Diagnostici, Italy) from 2003 up to now. The World Health Organization (WHO) N. gonorrhoeae O, M, and N reference strains were used for quality control.19 The MIC values were interpreted according to the EUCAST clinical breakpoint criteria (v. 7.1, 2017) (http://eucast.org/clinical_breakpoints/).

Antimicrobial susceptibility data for the PPNG belonging to the first time period (2003–2004) were previously described.14,20

Results

Patient characteristics

Of all the isolates selected for this study between 2003 and 2004, the 87.5% (35/40) were isolated from the urethra of males and the remaining endocervix. The median age was 35 years for both sexes. Among males who have declared their sexual orientation, 26 were men who have sex with women (MSW), whereas 3 were men who have sex with men (MSM). All the females declared to be heterosexual (data not shown).

In 2014–2015, the majority of isolates (38/40; 95%) were retrieved from male patients with a median age of 30.5 years. For two cases gender information was not available. Among the patients who declared their sexual orientation, 16 were MSW and 21 were MSM (data not shown).

Characterization of PPNG isolated in 2003–2004

blaTEM-135, blaTEM-1, and a new blaTEM, the blaTEM-228 (accession no.: KY851075), have been identified. The blaTEM-228 showed one nucleotide substitution at position 547 of the gene (G → A) resulting in a single amino acid substitution, A185T.

The blaTEM-135 in Toronto/Rio plasmid was the most frequent (23/40; 57.5%), followed by blaTEM-1 (16/40; 40%) and blaTEM-228 (1/40; 2.5%), both carried by African plasmid types (Table 1). Forty strains were assigned to 18 STs, with ST661 (19/40; 47.5%) being the predominant, followed by ST660 (3/40; 7.5%), ST655, and ST656 with two isolates each, and 14 STs
Penicillin MIC values ranged from 4 to >32 mg/L; 80% (32/40) were resistant to ciprofloxacin (MIC >0.06 mg/L) (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/mdr).

Characterization of PPNG isolated in 2014–2015

As shown in Table 1, \( \text{bla}_{\text{TEM}}-1 \), \( \text{bla}_{\text{TEM}}-135 \), and \( \text{bla}_{\text{TEM}}-228 \) have been detected. The \( \text{bla}_{\text{TEM}}-1 \) possessed by the African plasmid was the predominant type (21/40; 52.5%). The \( \text{bla}_{\text{TEM}}-135 \) allele (18/40; 45%) was located on Toronto/Rio (14/40; 35%) and Asian plasmids (4/40; 10%), whereas one isolate harbored \( \text{bla}_{\text{TEM}}-228 \) on the African plasmid. Forty strains were assigned to 21 STs, with ST5624 as the most frequent (12/40; 30%), followed by ST1582 (3/40; 7.5%). There were 2 isolates each of ST5793, ST9918, and ST13231, whereas 16 STs (ST661, ST995, ST1461, ST2886, ST3104, ST4995, ST5364, ST9541, ST11657, ST11658, ST13071, ST13228, ST13229, ST13230, ST13232, and ST13239) were all identified in only 1 isolate (Fig. 1). Five STs (ST13229, ST13230, ST13231, ST13232, and ST13239) were novel to the NG-MAST database.

In the period 2014–2015, the penicillin MIC values ranged from 6 to >32 mg/L; 75% (30/40) were resistant to ciprofloxacin (MIC >0.06 mg/L) (Supplementary Table S2).

Overall, none of the samples were resistant to ceftriaxone (MIC values ranging between 0.002 and 0.012 mg/L) or spectinomycin (MIC >64 mg/L) (Supplementary Tables S1 and S2).

Association among \( \text{bla}_{\text{TEM}} \) alleles, plasmid types, and NG-MAST

In 2003–2004, \( \text{bla}_{\text{TEM}}-135 \)/Toronto/Rio was mainly associated with ST661 (19/40; 47.5%), as well as ST655, ST657, and ST1479. Conversely, isolates harboring \( \text{bla}_{\text{TEM}}-1 \)/African belonged to 13 STs (ST69, ST331, ST656, ST658, ST659, ST660, ST662, ST663, ST665, ST666, ST669, ST1249, and ST1253). One isolate, related to ST667, harbored the \( \text{bla}_{\text{TEM}}-228 \)/African (Fig. 1).

In 2014–2015, ST5624 was the most frequent (12/40; 30%), of which nine harbored \( \text{bla}_{\text{TEM}}-135 \)/Toronto/Rio; two

<table>
<thead>
<tr>
<th>Plasmid type</th>
<th>( \text{bla}_{\text{TEM}}-1 )</th>
<th>( \text{bla}_{\text{TEM}}-135 )</th>
<th>( \text{bla}_{\text{TEM}}-228 )</th>
<th>( \text{bla}_{\text{TEM}}-14S )</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003–2004</td>
<td>16 (40.0%)</td>
<td>0 (0.0%)</td>
<td>1 (2.5%)</td>
<td>0 (0.0%)</td>
<td>17</td>
</tr>
<tr>
<td>African</td>
<td>0 (0.0%)</td>
<td>23 (57.5%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>23</td>
</tr>
<tr>
<td>Toronto/Rio</td>
<td>21 (52.5%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>1 (2.5%)</td>
<td>22</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (0.0%)</td>
<td>4 (10.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>41</td>
<td>1</td>
<td>0</td>
<td>80</td>
</tr>
</tbody>
</table>

FIG. 1. Distribution of \( \text{bla}_{\text{TEM}} \) allele/plasmid type and ST in 80 Neisseria gonorrhoeae isolates collected in Italy in 2003–2004 and 2014–2015. ST, sequence type.
were \( \text{bla}_{\text{TEM-135}}/\text{Asian} \); and one isolate possessed \( \text{bla}_{\text{TEM-1}}/\text{African} \) (Fig. 1).

A total of four STs (ST661, ST9541, ST9918, and ST13232) were also associated to \( \text{bla}_{\text{TEM-135}}/\text{Toronto/Rio} \). Two PPNG isolates belonging to ST4995 and ST11658 harbored \( \text{bla}_{\text{TEM-135}}/\text{Asian} \).

Conversely, the broadest diversity of STs (ST995, ST1461, ST1582, ST2886, ST3104, ST5793, ST11657, ST13071, ST13228, ST13229, ST13230, ST13231, and ST13239) was associated with isolates that harbored \( \text{bla}_{\text{TEM-1}}/\text{African} \). The \( \text{bla}_{\text{TEM}}/\text{P14S/African} \) was identified in only one isolate belonging to ST3634 (Fig. 1).

**Discussion**

Although penicillin is no longer used in clinical practice for the treatment of gonorrhea, the plasmid–mediated penicillin resistant gonococci (PPNG) have been widely described.1,5–9,11

According to previous studies,5,6 few mutations in \( \text{bla}_{\text{TEM-1}} \) and a single mutation in \( \text{bla}_{\text{TEM-135}} \) have been described to be more likely associated with extended-spectrum \( \beta \)-lactamase (ESBL) in \( \text{N. gonorrhoeae} \). This is considered a very relevant recent detection (2014–2015). The \( \text{bla}_{\text{TEM-135}} \) allele was ciprofloxacin resistant. In this scenario, overall the susceptibility for penicillin, ceftriaxone, and tetracycline resistance pattern. Conversely, ST5624 was more likely to be associated with ciprofloxacin resistance.

Similar to what was reported in England, Wales, and France,6,10 ST5624 was more likely to be associated with Toronto/Rio plasmid harboring \( \text{bla}_{\text{TEM-135}} \). In this scenario, overall the susceptibility for penicillin, ceftriaxone, and tetracycline resistance pattern. Conversely, ST5624 was associated with gonococci of recent isolation and mainly characterized by ciprofloxacin resistance.

Of note, all the PPNG isolated in 2014–2015 were included in the study, whereas the PPNG of the first period, 2003–2004, represented around 50% of the total. The different percentages of PPNG between the two periods (53% vs. 9.2%) could be, in part, explained by a change in the molecular characteristics of the gonococcal populations over time in terms of plasmid–mediated resistance capability.

A different trend of isolates carrying plasmid type and \( \text{bla}_{\text{TEM}} \) gene was observed. In particular, Toronto/Rio harboring \( \text{bla}_{\text{TEM-135}} \) decreased from 57.5% in 2003–2004 to 35% in 2014–2015. On the contrary, African plasmid possessing \( \text{bla}_{\text{TEM-1}} \) increased from 42.5% in 2003–2004 to 55% in 2014–2015.

The decrease of isolates carried by Toronto/Rio plasmid that possessed \( \text{bla}_{\text{TEM-135}} \) allele and the parallel increase of those with African plasmid carrying \( \text{bla}_{\text{TEM-1}} \) suggest a change in the molecular plasmid profile of PPNG circulating in the country. Furthermore, compared to Toronto/Rio and Asian plasmids, the African plasmid exhibits a broad diversity of \( \text{bla}_{\text{TEM}} \) alleles.

ST661 and ST5624 were prevalent in the two periods, respectively, even though it observed a higher variability among PPNG of more recent isolation. As previously described,14,20 gonococci belonging to ST661 have been recovered up to 2007 and were characterized by ciprofloxacin, penicillin, and tetracycline resistance pattern. Conversely, ST5624 was associated with gonococci of recent isolation and mainly characterized by ciprofloxacin resistance.

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