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Original article

Molecular epidemiology of penicillinase-producing *Neisseria gonorrhoeae* isolates in France

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ABSTRACT

Objectives: Characterizing the molecular epidemiology of antibiotic resistance is crucial for a better understanding of the evolution and spread of resistance in *Neisseria gonorrhoeae*. Here, we examine the molecular epidemiology of penicillinase-producing *N. gonorrhoeae* (PPNG) isolates in France.
Methods: We investigated 176 PPNG isolates collected between 2010 and 2012 by the National Reference Centre in France. Genotyping was performed using the NG-MAST technique, *bla*$_{TEM}$ genes were Sanger-sequenced, and plasmids were characterized by PCR-typing.
Results: We revealed the existence of four major clusters representing about one-third of PPNG circulating in France. These clusters were related to ST1479 (18/176, 10.2%), to ST1582 (15/176, 8.5%), to ST8922 (10/176, 5.6%), and to ST1285 (9/176, 5.1%). Wild-type TEM-1 was identified in 151 (151/176, 85.8%) PPNG isolates, and TEM-1 variants were mostly represented by the M182T mutation (14/176, 8%), followed by P14S/L (8/176, 4.5%), G228S (2/176, 1.1%), and Q269K (1/176, 0.6%). The *bla*$_{TEM}$ genes were carried by African (157/176, 89.2%), Asian (13/176, 7.4%), and Toronto/Rio (6/176, 3.4%) plasmids. The M182T variants were found in various genetic backgrounds, whereas the P14S variants were disseminated clonally. The G228S and Q269K variants belong to one of the four major clusters of PPNG, which suggests a recent *de novo* emergence of these mutations.
Conclusions: Our results show that approximately one-third of the penicillinase-producing *N. gonorrhoeae* isolates in France belong to one of four major clusters and that the spread of the different TEM variants is associated with distinct patterns of molecular epidemiology. **M. Micaëlo, Clin Microbiol Infect 2017;23:968**

Introduction

Neisseria gonorrhoeae is one of the most prevalent sexually transmitted bacteria and remains a major public health problem [1]. Sexually transmitted infections caused by *N. gonorrhoeae* are increasing with the global emergence of multidrug-resistant isolates, including those resistant to β-lactam antibiotics [2]. *N. gonorrhoeae* is naturally susceptible to β-lactam antibiotics, and penicillin-resistant isolates can be explained by two major mechanisms: one is based on the modification of penicillin binding proteins (PBPs), which are the target of β-lactam antibiotics, and the other involves the production of TEM-1 β-lactamase, which hydrolyses penicillins [3,4]. Resistance to third-generation cephalosporin is more complex and mainly results from multiple mutations in the PBP genes, which generate mosaic PBPs with a poor affinity for cephalosporins [5].

The first TEM-1-producing strain was isolated in the USA in 1976 [6]. The *bla*$_{TEM-1}$ gene in this isolate was carried by a 7.4 kb-plasmid,

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called pJD4, or Asian plasmid. The bla_{TEM-1} gene has been widely described on plasmids which derive from Asian plasmid but differ in size: particularly, African plasmid, pJD5 (5.6 kb), and Toronto/Rio-type plasmid, pJD7 (5.2 kb) [7–9].

Recent studies have demonstrated the emergence of TEM-135, a variant of TEM-1 harbouring the M182T stabilizing mutation, in Asia [10–12], Australia [9], Argentina [13], and the UK [14]. Interestingly, this variant has been described in WHO European, African, American, Southeast Asian, and Western Pacific regions in a large collection of isolates [15]. However, most of these studies focused on TEM-135 using an allele-specific PCR approach, and only a few studies have fully explored the TEM diversity in *N. gonorrhoeae* clinical isolates [14,15]. The goal of our work was to define the molecular epidemiology of TEM-producing *N. gonorrhoeae* isolates in France.

Materials and methods

N. gonorrhoeae isolates and antimicrobial susceptibility testing

In France, the epidemiological surveillance of gonorrhoea is based on two voluntary sentinel networks that rely on either clinicians (the ResIST network) or public and private laboratories (the Renago network). The 3618 *N. gonorrhoeae* isolates included in this study were collected from December 2010 to October 2012 by the Renago network from the National Reference Centre for Gonococci in Paris, France [16]. Of these 3618 *N. gonorrhoeae* isolates, 309 (8.5%) penicillinase-producing *N. gonorrhoeae* (PPNG) isolates were identified using oxoid nitrocefin disks (Oxoid, Dardilly, France). Of these 309 PPNG isolates, 176 were randomly selected for further microbiological and molecular studies.

The MICs of penicillin, cefixime, ceftriaxone, ciprofloxacin, tetracycline, and spectinomycin were determined using the E-test method (i2A, Montpellier, France; Biomerieux, Marcy l'Etoile, France) on chocolate agar plates, and the interpretation was performed according to the interpretative criteria of EUCAST [17].

Molecular procedures

Extraction of genomic DNA was performed using the Matrix InstaGene kit (BioRad, Marnes-La-Coquette, France) according to the manufacturer's recommendations. PCR/sequencing of the bla_{TEM} gene was performed using the OT1/OT2/OT3/OT4 primers as previously described [18]. Plasmid typing for discriminating Asian, African, and Toronto/Rio plasmids was performed using the primers JDA and JDB, as previously described [19], and *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) was performed by sequencing internal fragments of two highly polymorphic loci, *porB* and *tbpB*, as previously described [20,21].

The edited and trimmed sequences were uploaded to the publicly accessible NG-MAST database (www.ng-mast.net) to obtain the allele number of each gene and the sequence type (ST). A maximum-likelihood phylogenetic tree was constructed using PHYML software [22] with the concatenated *porB* and *tbpB* alleles. Clusters were defined for isolates with <2% variation in concatenated sequences.

Ethical considerations

All of the procedures were performed in accordance with the ethical standard of the Helsinki declaration of 1975, revised in 2000. Demographical data (including geographical origin used in this study) were anonymized, and the database was approved and authorized at a national level (Commission Nationale Informatique et Liberté, n° 718544).

Results

The 176 PPNG isolates were fully susceptible to third-generation cephalosporins (MIC to cefixime and to ceftriaxone ranging from 0.016 mg/L to 0.064 mg/L and from 0.002 mg/L to 0.032 mg/L, respectively) (Fig. S1, supporting information). Furthermore, 80.7% (142/176) of the isolates were resistant to ciprofloxacin, and 98.9% (174/176) were resistant to tetracycline, with 75% (132/176) demonstrating a high level of resistance to tetracycline (MIC>12 mg/L). All isolates were susceptible to spectinomycin.

Genotyping of penicillinase-producing N. gonorrhoeae isolates

The 176 penicillinase-producing *N. gonorrhoeae* isolates belonged to 106 NG-MAST sequence types (STs), of which 48 STs (45%) were not previously described. Of the 176 PPNG isolates, 81 isolates were singletons. Using the concatenate of *porB* and *tbpB* sequences, we revealed four major clusters of PPNG circulating in France. The two major clusters were cluster A, which contained 18/176 isolates (10.2%) related to ST1479, and cluster B, which contained 15/176 isolates (8.5%) related to ST1582. The third and fourth clusters were defined by sequence type ST8922 (10/176; 5.7%) and ST1285 (9/176; 5.1%), respectively (Fig. 1). The distribution of the four major clusters varies by region in France, as shown in Fig. 2 and Table S1 (North East (NE), South East (SE), North West (NW), South West (SW) and Paris area (PA)).

Mutations in the TEM-1 protein

All of the 176 penicillinase-producing *N. gonorrhoeae* isolates harboured a bla_{TEM} gene. Six variants of TEM-1 were identified (Table 1). Wild-type TEM-1 was identified in 151/176 (85.7%) of the PPNG isolates, whereas TEM-135, which harbours the M182T stabilizing mutation, was identified in 14/176 isolates (8.0%). Among the 11 remaining isolates, eight harboured a mutation in the signal peptide of TEM-1 at position 14 (P14S, $n = 7$ and P14L, $n = 1$), two isolates contained a point-mutation at position 228 (G228S), and one contained a point-mutation at position 269 (Q269K).

Except for the missense mutations, the nucleotide sequence backbone corresponding to bla_{TEM1b} was the same for all of the isolates.

Diversity of plasmids carrying the bla_{TEM} gene

The results of the plasmid typing PCR on the 176 PPNG isolates showed that the African plasmid was the most common (157/176; 89.2%), followed by the Asian plasmid (13/176; 7.4%) and the Toronto/Rio plasmid (6/176; 3.4%) (Table 1). All of the Toronto/Rio plasmids (6/6) and 61.5% (8/13) of the Asian plasmids contained the $bla_{TEM-135}$ gene, whereas 93.6% (147/157) of the African plasmids contained the bla_{TEM-1} allele. The bla_{TEM} alleles encoding the G228S and Q269K variants were carried by African plasmids. Of those carrying a mutation in the signal peptide ($n = 8$), seven were carried by an African plasmid and one by an Asian plasmid.

Discussion

Since the first description of gonococcal resistance to penicillins via the production of a TEM β-lactamase, both molecular studies [3,7] and surveillance network data have shown that the resistance frequencies can vary by country. According to ECDC, the rate of PPNG isolates varied from 2% to 31.6% in Europe in 2011 [23], and different studies have described a large range, from 1.4% in Japan [10] and 4.6% in England and Wales [14] to approximately 20% in Argentina [13] and 40% in China [12]. In our work, 8.5% (309/3618)

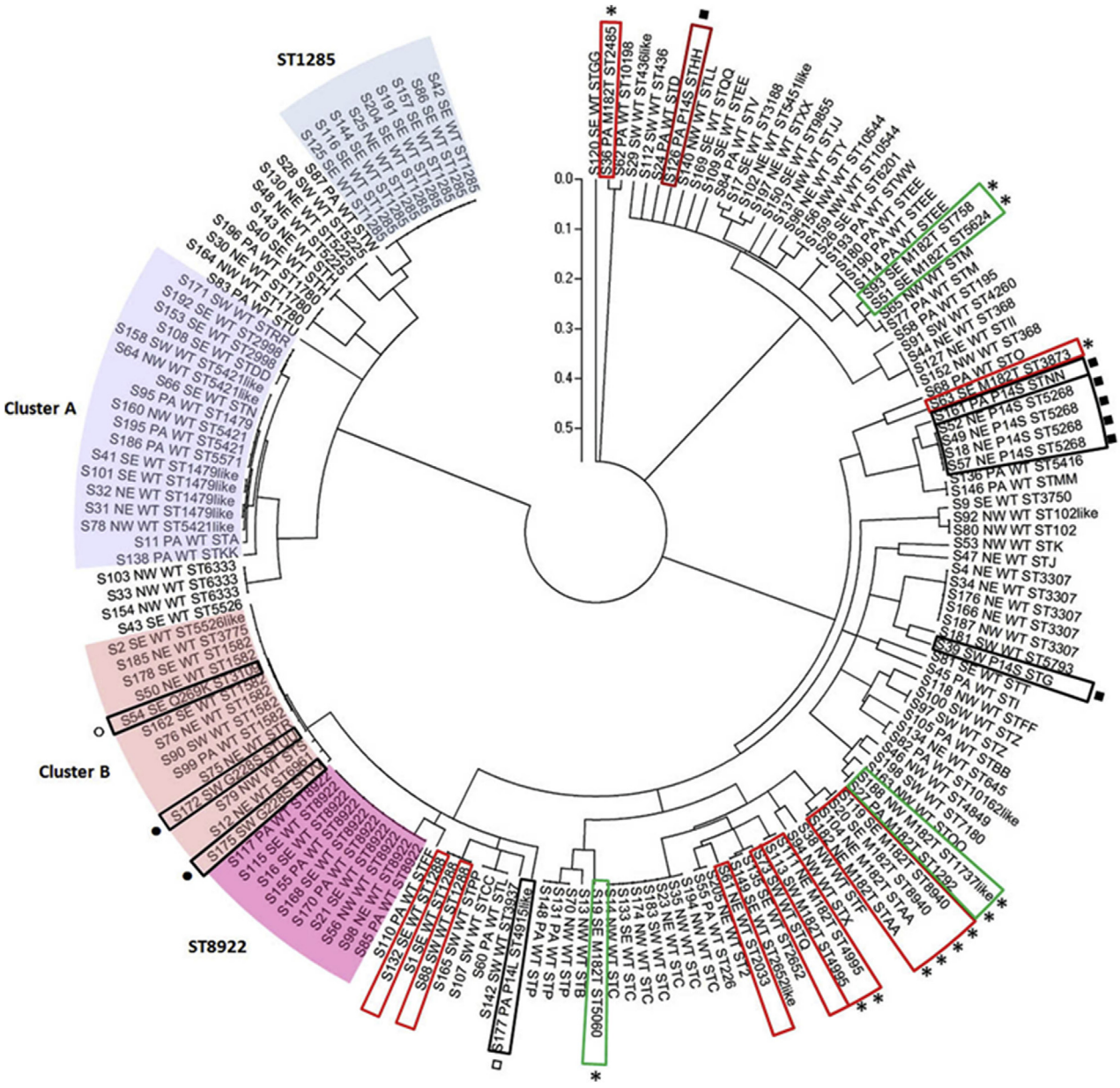

Fig. 1. Phylogenetic tree of concatenated *porB* and *tbpB* sequences of PPNG isolates. Each isolate is annotated with its ID number, its region of origin (NW, North West; NE, North-East; SW, South West; SE, South-East; PA, Paris Area), the TEM mutation (WT = wild type), and NG-MAST ST. The main STs and clusters (ST8922 and ST1285, cluster A and cluster B) are highlighted with light colors. Isolates harbouring an Asian plasmid are boxed in red, a Toronto/Rio plasmid in green, and an African plasmid with a TEM variant in black. The non-boxed isolates harbour an African plasmid associated with TEM-1. The TEM variants are indicated with the following symbols: □: P14L, ■: P14S, *: M182T, ●: G228S, ○: Q269K.

of the *N. gonorrhoeae* isolates collected by the Renago national surveillance network between 2010 and 2012 were PPNG. The reasons for these differences are not clearly understood, thus underlining the importance of molecular epidemiology studies. Here, we carried out the first molecular epidemiology study of PPNG in France, in which we measured the diversity of the bla_{TEM} alleles, identified their plasmid vectors, and determined the genetic background of the *N. gonorrhoeae* isolates using the NG-MAST genotyping method.

The PPNG isolates circulating in France belong to many different STs, most of which have never been described. This observation corroborates the diversity and emergence of new STs described in previous studies focusing on the epidemiology of PPNG isolates [9,11–15]. Moreover, the STs that we identified were mostly different from those described in Europe [14,15], South America [13], Asia [12], and Australia [9]. Here, we found that 29.5% (52/176) of isolates belonged to four major clusters related to either ST1479 and ST1582 (10.2% and 8.5%, respectively), or to ST8922 and ST1285 (5.6% and 5.1%, respectively) (Fig. 1).

If we focus on the distribution of these main clusters in different regions, frequency variations are apparent (Fig. 2) that probably reflect the local transmission of these isolates.

Concerning the plasmids harbouring the bla_{TEM} gene, African plasmid was the most common (89.2%), followed by Asian (7.4%) and Toronto/Rio (3.4%) plasmids (Table 1). Previous studies highlighted African plasmid as the most prevalent plasmid in PPNG isolates, with representation between 72.5% and 82.3% [9,13,14], but various abundances of Toronto/Rio plasmid have been described, with a prevalence of 10% in Thailand and UK [11,14], 13% in Australia [9], and 26.6% in Argentina [13]. This difference in Toronto/Rio

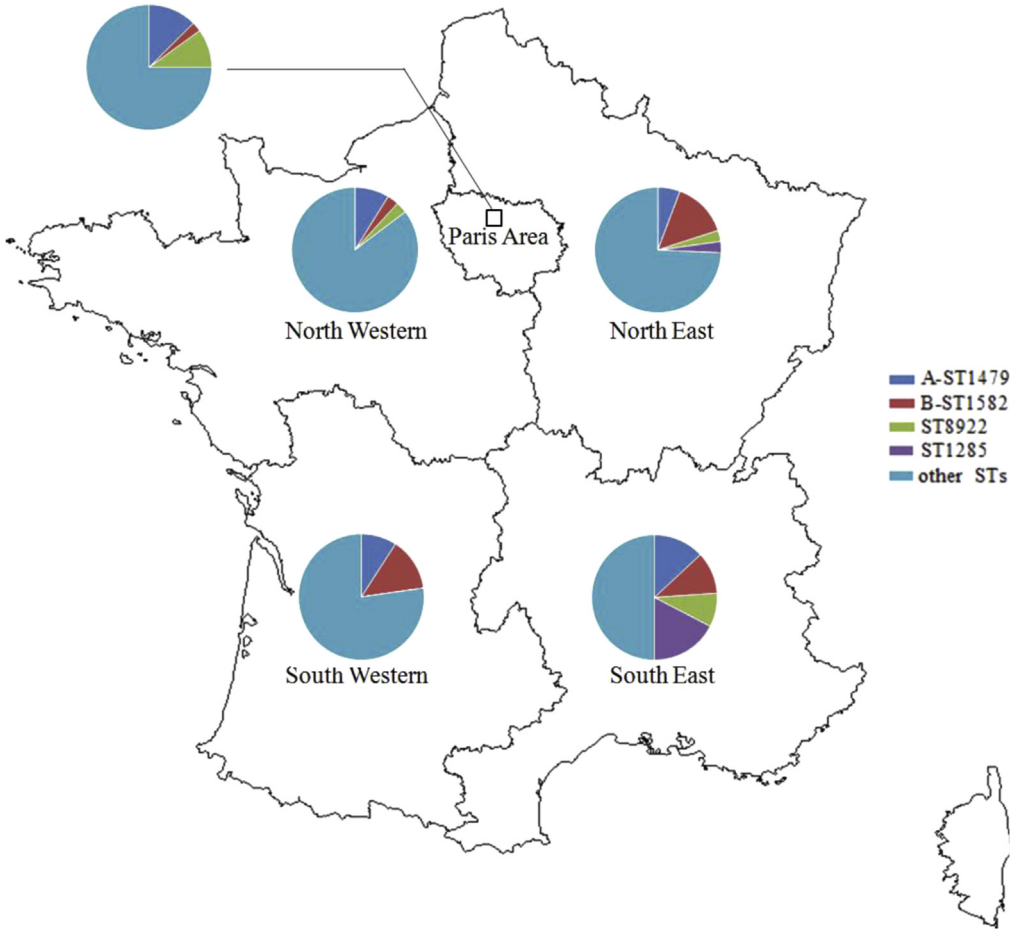

Fig. 2. Distributions of penicillinase-producing *N. gonorrhoeae* isolates in France, by region.

Table 1
Characterization of the TEM-1 variants and their plasmid location in the penicillinase-producing *N. gonorrhoeae* isolates

Plasmid types	Aminoacid mutation in TEM-1 β-lactamase						
	Wild type	P14L	P14S	M182T (TEM-135)	G228S	Q269K	Total
pJD4 (Asian)	4	–	1	8	–	–	13 (7.4%)
pJD5 (African)	147	1	6	–	2	1	157 (89.2%)
pJD7 (Toronto/Rio)	–	–	–	6	–	–	6 (3.4%)
Total	151 (85.8%)	1 (0.6%)	7 (4.0%)	14 (8.0%)	2 (1.1%)	1 (0.6%)	176 (100%)

plasmid prevalence is of particular interest in TEM-135 epidemiology because Toronto/Rio plasmid is quasi-systematically associated with $bla_{\text{TEM-135}}$ [9,13–15].

In our study, we found 8% (14/176) of the PPNG isolates to be harbouring the M182T variant of TEM-135 (Table 1), whereas previous studies found higher frequencies of this variant: 10% in Thailand [11], approximately 27% in Argentina, Australia, and the UK, [9,13,14] and 58% in China [12]. In the studies carried out in Thailand and Argentina, TEM-135 was mainly harboured by a Toronto/Rio plasmid [11,13], whereas the studies conducted in Australia and UK found a higher prevalence of African and Asian plasmids [9,14]. Here, we found that the $bla_{\text{TEM-135}}$ gene was embedded in both the Toronto/Rio (6/14, 42.9%) and Asian (8/14, 57.1%) plasmids. These results underline the diversity of M182T variant emergence, which is not only mediated by Toronto/Rio plasmid but is also emerging and spreading in other plasmids. Concerning the genetic background in which the TEM-135 plasmids were identified, we found various STs belonging to rare clusters (Fig. 1). In previous studies, local spread of clones has been described [9,11–14] without corresponding evidence to support the international spread of a given clone. Cole et al. found a majority of ST4995 (58%) among the Asian/TEM-135 isolates in UK [14], and we identified two of the eight tested Asian/TEM-135 isolates belonging to this ST in our study. Similarly, Cole et al. described two of seven Toronto/Rio/TEM-135 isolates belonging to ST5624, and we found one of six Toronto/Rio/TEM-135 isolates belonging to this ST. Hence, a molecular epidemiological comparison between France and UK allowed us to highlight the diffusion of TEM-135 clones in isolates carrying both the Toronto/Rio and Asian plasmids.

In our study, 4.0% (7/176) of the PPNG isolates harboured the P14S variant versus 18.7% (14/75) in Cole's study (Table 1 and Fig. 1) [14]. Most of these isolates (4/7 in our work and 10/14 in Cole's study) were associated with an African plasmid and a ST5268 background [14]. Here again, the emergence of the P14S variant in France and the UK is highly associated with clonal spread.

The G228S ($n = 2$) and Q269K ($n = 1$) variants were identified on African plasmids in the second most prevalent cluster of PPNG isolates (cluster B related to ST1582 (Fig. 1)). Hence, the G228S and Q269K variants were described as emerging from TEM-1 in the most prevalent plasmid (African) in a highly circulating cluster in France, which suggests the *de novo* emergence of these mutations.

The diversity of TEM mutations in *N. gonorrhoeae* isolates compared with those found in *Enterobacteriaceae* isolates (http://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources) suggests that TEM evolution does not follow the same pathway in these bacteria. Hence, M182T has been described as a stabilizing mutation [24] that acts as a secondary compensatory mutation when it is associated with other ESBL-conferring mutations [25]. Therefore, this mutation on its own probably does not confer a selective advantage in *Enterobacteriaceae* isolates and, as such, is rarely described alone [26]. Surprisingly, this mutation was found in up to 58% of the *N. gonorrhoeae* isolates [12]. If the primary selection of such a stabilizing mutation is found in *N. gonorrhoeae*, we can assume that stability likely acts with a higher selective pressure in *N. gonorrhoeae* than in *Enterobacteriaceae*. Interestingly, we have previously described that the pre-existence of the M182T mutation greatly modifies the adaptive landscape of TEM-1 [27]. This finding suggests that TEM-135 can accumulate more mutations, thus opening the door to ESBL-conferring mutations in *N. gonorrhoeae*. To date, resistance to third-generation cephalosporins is mainly caused by mutations in the PBP genes. The evolutionary perspective of such TEM mutants highlights the importance of epidemiological surveillance of PPNG.

Similarly, different mutations have been identified in the signal peptide at position P14 in *N. gonorrhoeae* that have never been identified in *Enterobacteriaceae*. The P14S, P14L, and P14T variants have been described in this study or previously [14,15], which suggests that this residue is under selection in *N. gonorrhoeae*. This may reflect an adaptation of the TEM signal peptide to the secretion system in *N. gonorrhoeae*. The G228S mutation, which is located in the loop of the enzyme distant from active site, was identified both in our study and in a previous worldwide study [15], suggesting that this is an adaptive mutation rather than a polymorphism.

In conclusion, we have shown that approximately one-third of the TEM-producing *N. gonorrhoeae* isolates in France belong to one of four major clusters, and we have demonstrated that the spread of different TEM β-lactamase variants is associated with distinct patterns of molecular epidemiology. Clones harbouring the M182T and P14S variants were identified in both the UK and France, highlighting that international surveillance programmes are essential for understanding and controlling the spread of *N. gonorrhoeae* isolates.

In our study, the analysis of genetic determinants was limited to *porB*/*tbpB* sequencing and to plasmid diversity using PCR-typing. Using whole genome sequencing and robust epidemiological data should have probably given a better understanding of penicillinase-producing *N. gonorrhoeae* epidemiology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2017.04.010.

Transparency declaration

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