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Genetic diversity of *bla*$_{TEM}$ alleles, antimicrobial susceptibility and molecular epidemiological characteristics of penicillinase-producing *Neisseria gonorrhoeae* from England and Wales

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Objectives: The objective of this study was to investigate the genetic diversity of *bla*$_{TEM}$ alleles, antimicrobial susceptibility and molecular epidemiological characteristics of penicillinase-producing *Neisseria gonorrhoeae* (PPNG) isolates collected in 2012 from England and Wales.

Methods: PPNG isolates were from the 2012 Gonococcal Resistance to Antimicrobial Surveillance Programme (GRASP). Their susceptibility to seven antimicrobials was determined using agar dilution methodology. β-Lactamase production was detected using a nitrocefin test. β-Lactamase plasmid types were determined and *bla*$_{TEM}$ genes were sequenced. Isolates were also typed by *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST).

Results: Seventy-three PPNG isolates were identified in the 2012 GRASP collection (4.6%, 73/1603). Three different *bla*$_{TEM}$ alleles were identified, encoding three TEM amino acid sequences: TEM-1 (53%), TEM-1 with a P14S substitution (19%) and TEM-135 (27%). The *bla*$_{TEM-135}$ allele was present in nine different NG-MAST types and was found mostly on Asian (60%) and Toronto/Rio (35%) plasmids. By contrast, most TEM-1-encoding plasmids were African (98%). All the TEM-135 isolates displayed high-level ciprofloxacin and tetracycline resistance.

Conclusions: The high proportion of *bla*$_{TEM-135}$ alleles (27%) demonstrates that this variant is circulating within several gonococcal lineages. Only a single specific mutation near the β-lactamase active site could result in TEM-135 evolving into an ESBL. This is concerning particularly because the TEM-135 isolates were associated with high-level ciprofloxacin and tetracycline resistance. It is encouraging that no further TEM alleles were detected in this gonococcal population; however, vigilance is vital as an ESBL in *N. gonorrhoeae* would render the last remaining option for monotherapy, ceftriaxone, useless.

Introduction

Gonorrhoea is a major public health concern in England and Wales due to the increasing numbers of new cases (e.g. from 25577 in 2012 to 29291 in 2013)[1] and the ability of the causative agent, *Neisseria gonorrhoeae*, to develop resistance to all antimicrobial agents used for treating gonorrhoea.[2] Penicillinase-producing *N. gonorrhoeae* (PPNG) strains were first detected in 1976 in both the UK[3] and the USA.[4] The rapid dissemination of PPNG strains with high-level, plasmid-mediated penicillin resistance, combined with emergence of chromosomally mediated penicillin resistance, rendered this once highly effective antimicrobial abandoned in the USA by 1987.[5] PPNG strains continue to circulate globally.[6–8] In 2012, the total penicillin resistance (chromosomally mediated and plasmid-mediated resistance) in gonococci in England and Wales, as determined by the Gonococcal Resistance to Antimicrobial Surveillance Programme (GRASP), was 14.6% and PPNG prevalence was 4.6%.[9] This PPNG level is low compared with many countries in, e.g. South-East Asia, where PPNG rates may be as high as 100%.[6]

PPNG strains traditionally produce TEM-1 β-lactamase, with the encoding *bla*$_{TEM}$ gene located on one of seven different plasmids,[10] all of which share a high degree of sequence homology.[11] This TEM-1 penicillinase hydrolyses penicillins, but is not active against extended-spectrum cephalosporins, such as cefixime and ceftriaxone, which are currently used for gonorrhoea treatment[12] and are the last options for empirical antimicrobial monotherapy.[2] The TEM-135 enzyme, which differs from TEM-1 by one

amino acid substitution (M182T), was first identified in *Salmonella enterica* subsp. *enterica* serovar Typhimurium from a rabbit in Italy,[13] but has been recently described in *N. gonorrhoeae* in several studies.[10,14–20] A recent study found that TEM-135 has been present in the Toronto/Rio plasmid in *N. gonorrhoeae* since at least 1984.[10] TEM-135 also lacks ESBL activity. However, the M182T substitution increases enzyme stability[21] and only one additional change at any one of several key amino acid positions would be needed to confer capacity to hydrolyse extended-spectrum cephalosporins. A non-ESBL β-lactamase, TEM-220, with both the M182T and an A185T substitution has recently been identified in a *N. gonorrhoeae* strain isolated in Argentina.[19] ESBLs are prevalent worldwide in the Enterobacteriaceae and the increasing prevalence of ESBL-producing *Escherichia coli* in the community setting provides further opportunities for *N. gonorrhoeae*, a naturally transformable species, to be exposed to ESBL-encoding genes in the uro-genital tract.

We investigated the genetic diversity of bla_{TEM} alleles, antimicrobial susceptibility and molecular epidemiological characteristics of PPNG isolates collected in England and Wales, to identify the proportion and characteristics of TEM-1 and TEM-135 isolates and to seek any circulating isolates with additional bla_{TEM} alleles that may have emerged due to the use of extended-spectrum cephalosporins to treat gonorrhoea.

Materials and methods

Isolates submitted to GRASP in 2012[9] were tested for susceptibility to penicillin G, ciprofloxacin, spectinomycin, azithromycin, tetracycline, ceftriaxone and cefixime using the GRASP agar dilution method.[22] The Oxoid nitrocefin solution test (Oxoid, Basingstoke, UK) was used to detect β-lactamase production.

The β-lactamase plasmid types were determined by PCR[23] and the bla_{TEM} genes were amplified and sequenced as previously described.[15] *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) was performed as previously described[24] and the NG-MAST STs were established at www.ng-mast.net. All sequence analysis was performed using Bionumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium) and visualization of phylograms was performed in FigTree version 1.4.2 (A. Rambaut, University of Edinburgh, UK).

Geometric means of the MICs were calculated for three *N. gonorrhoeae* categories (non-PPNG, PPNG with TEM-1 and PPNG with TEM-135). Linear regression was used to study the relationship between the MICs and the three different groups, as well as the relationship between TEM-1 and TEM-135 isolates when the median MICs for the isolate groups differed by at least two dilutions.

Results

Seventy-three PPNG isolates from 73 different episodes of infection were identified through GRASP in 2012 (4.6%, 73/1603). Fifty-eight PPNG isolates were from men (79.5%), 15 were from women (20.5%) and the median age of patients with a PPNG isolate was 28 years (range 18–70 years). Three different bla_{TEM} alleles were identified encoding two different mature penicillinases. Fifty-three (73%) isolates produced TEM-1 enzyme, 39 (53%) with the classical allele and 14 (19%) with a variant that differed from TEM-1 by one amino acid, in the signal peptide region at position 14 [bla_{TEM-1} (P14S)].[25] Twenty PPNG (27%) isolates produced TEM-135 (Figure 1).

MICs of penicillin G for all the PPNG isolates ranged from 1 to >4 mg/L and were significantly higher than the penicillin MICs for the non-PPNG isolates ($P<0.001$) (Table 1). The ciprofloxacin and tetracycline MICs for the TEM-135 isolates were significantly higher ($P<0.001$) than those for the TEM-1 isolates and the non-PPNG isolates (Table 1). For the remaining antimicrobials, the median and modal MICs for the TEM-135 isolates were either the same or one MIC dilution higher than for isolates with TEM-1 (Table 1).

The $bla_{TEM-135}$ allele was harboured on African, Asian and Toronto/Rio plasmids (Figure 1). Four different NG-MAST STs were identified in each of the TEM-135 PPNG groups with the Asian and Toronto/Rio plasmids. The PPNG with TEM-135 on an African

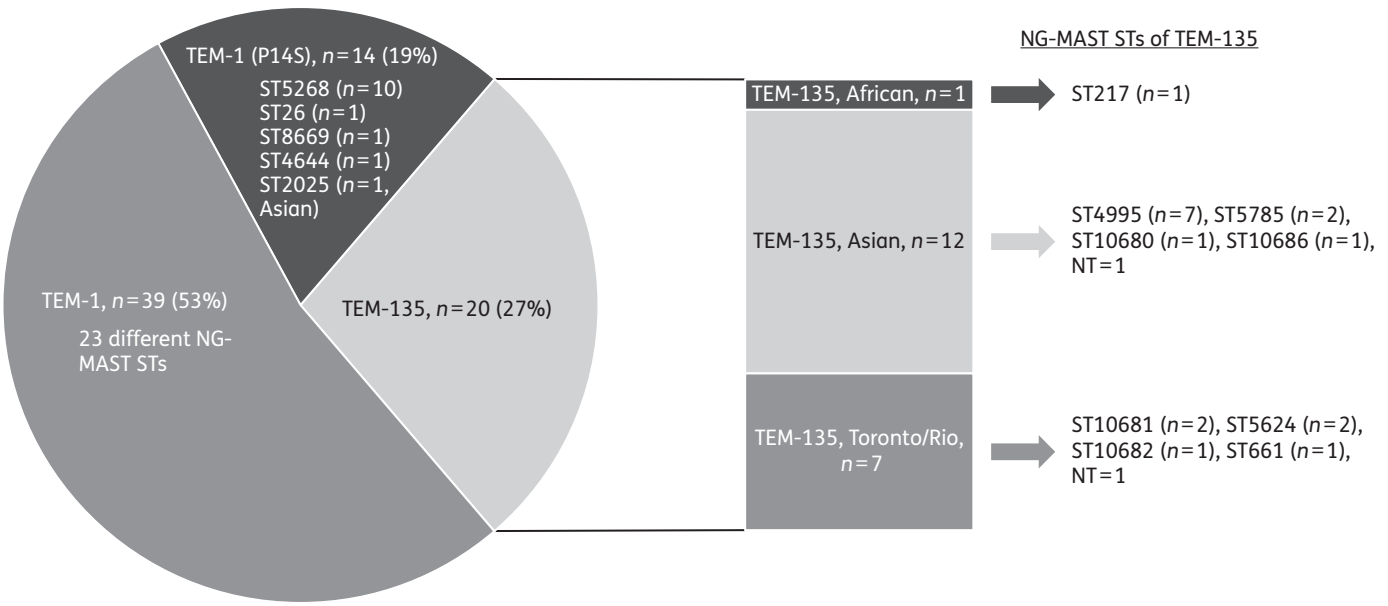

Figure 1. TEM enzyme distribution, β-lactamase plasmid type and NG-MAST STs of PPNG isolates from England and Wales (2012). NT, non-typeable.

Table 1. Minimum, maximum, mode, median and geometric mean MICs (mg/L) for the non-PPNG and PPNG with TEM-135 or TEM-1 in England and Wales, 2012

Parameter	Penicillin			Ceftriaxone			Cefixime			Ciprofloxacin			Azithromycin			Spectinomycin			Tetracycline		
	non-PPNG	TEM-1	TEM-135	non-PPNG	TEM-1	TEM-135	non-PPNG	TEM-1	TEM-135	non-PPNG	TEM-1	TEM-135	non-PPNG	TEM-1	TEM-135	non-PPNG	TEM-1	TEM-135	non-PPNG	TEM-1	TEM-135
Minimum	≤0.06	1	4	≤0.002	≤0.002	≤0.002	≤0.002	≤0.002	0.004	≤0.03	≤0.03	4	≤0.03	≤0.03	≤0.03	≤8	≤8	16	≤0.5	1	4
Maximum	2	>4	>4	0.125	0.03	0.015	0.25	0.03	0.125	>16	16	>16	>256	0.25	0.5	64	32	32	>16	>16	>16
Mode	0.25	>4	>4	0.004	≤0.002	0.004	0.008	0.004	0.004	≤0.03	≤0.03	8	0.125	0.06	0.125	16	16	32	4	>16	>16
Median	0.25	>4	>4	0.004	≤0.002	0.004	0.008	0.008	0.008	≤0.03	≤0.03	8	0.125	0.06	0.125	16	16	32	4	4	>16
Geometric mean[a]	0.218	3.331	4	0.005	0.003	0.005	0.011	0.007	0.009	0.128	0.219	8.574	0.106	0.078	0.128	19.54	19.804	26.909	3.052	5.334	12.553
P value (non-PPNG as reference)	—	**<0.001**	**<0.001**							—	0.134	**<0.001**							—	**<0.001**	**<0.001**
P value (TEM-1 as reference)											—	**<0.001**								—	**<0.001**

Number of observations: non-PPNG, 1530; TEM-1, 53; and TEM-135, 20.
P value from the linear regression model—model used only when the median MICs for the isolate groups differed by at least two dilutions to account for variations in MIC testing.
Bold values: significant ($P < 0.05$).
[a]Any MICs with 'less than' or 'greater than' values were converted into 'equal to' values, e.g. all ceftriaxone MICs ≤0.002 were categorized as 0.002 and all ciprofloxacin MICs >32 were categorized as 32.

plasmid belonged to one NG-MAST ST (Figure 1). Other than an Asian plasmid found in one isolate, the TEM-1-encoding plasmids were African, in gonococcal isolates belonging to 27 different NG-MAST STs (Figure 1). No overlap was seen among NG-MAST STs of isolates with TEM-1 or TEM-135 penicillinases (Figure 1). A phylogram created by NG-MAST concatenated *porB* and *tbpB* alleles displayed two main clades; the smaller clade represented isolates of the PorB1a (WI) serogroup, while the larger clade represented isolates of the PorB1b (WI/III) serogroup. The phylogram additionally revealed that each individual NG-MAST ST from the TEM-135 isolates (Figure 2) was ≥14 bp (1.6%) different from its nearest 'neighbours' (Figure 2). According to a previously used definition of NG-MAST genogroups (one of the two alleles identical and the other ≥99% similar),[26] this suggests that many of the TEM-135 isolates were not closely related.

Discussion

This is the first study, to our knowledge, to reveal the genetic diversity of bla_{TEM} alleles and molecular epidemiological characteristics of PPNG strains circulating in England and Wales. TEM-1 penicillinase was the most common (found in 73% of the PPNG isolates). However, the $bla_{TEM-135}$ allele was identified in several gonococcal lineages and three plasmid types, with the Asian plasmid type predominating. These data differ from studies performed in Thailand,[16] Argentina,[19] Australia[20] and a global study,[10] which all showed the Toronto/Rio plasmid to be the most prevalent carrier of $bla_{TEM-135}$. In accordance with previous studies, we found that TEM-1 was predominantly encoded by the African plasmid type,[10,16,19,20] the Toronto/Rio plasmid did not harbour the bla_{TEM-1} allele as was demonstrated in all but one[16] of the aforementioned studies, and we also detected an association between NG-MAST STs, the TEM enzyme and plasmid type.[15,16,18,19]

Compared with the TEM-1 and non-PPNG isolates, the TEM-135 isolates were all strikingly resistant to ciprofloxacin, whereas other studies have not reported any significant difference between the MICs of antimicrobials tested and the TEM-1 and TEM-135 isolates.[10,15,19] These UK TEM-135 isolates harboured three different main plasmid types and represented dissimilar NG-MAST STs, so the association with ciprofloxacin resistance is interesting, although the number of TEM-135 isolates in this study was quite small. WGS of these TEM-135 isolates might elucidate any genetic relatedness between these TEM-135 isolates that could not be distinguished from NG-MAST and plasmid typing.

Amino acid substitutions near the active site can increase the activity of TEM enzymes by enlarging the active site and thereby increasing the ability to hydrolyse extended-spectrum cephalosporins, which have larger oxyimino side chains. These substitutions can come at a cost of destabilizing the enzyme and lowering penicillinase activity.[27,28] It was originally proposed that M182T is always a secondary 'stabilizing' mutation or a 'suppressor' of substitutions that decrease enzyme stability or disrupt folding,[21,28,29] because until TEM-135 was first described,[13] the M182T substitution was only found in combination with other amino acid substitutions. Studies that have created M182T mutants have revealed either slightly lower ampicillin resistance/specific activity[28,29] or no difference in activity;[27] however, the presence of M182T in addition to substitutions at single and multiple positions results in restored activity and increased

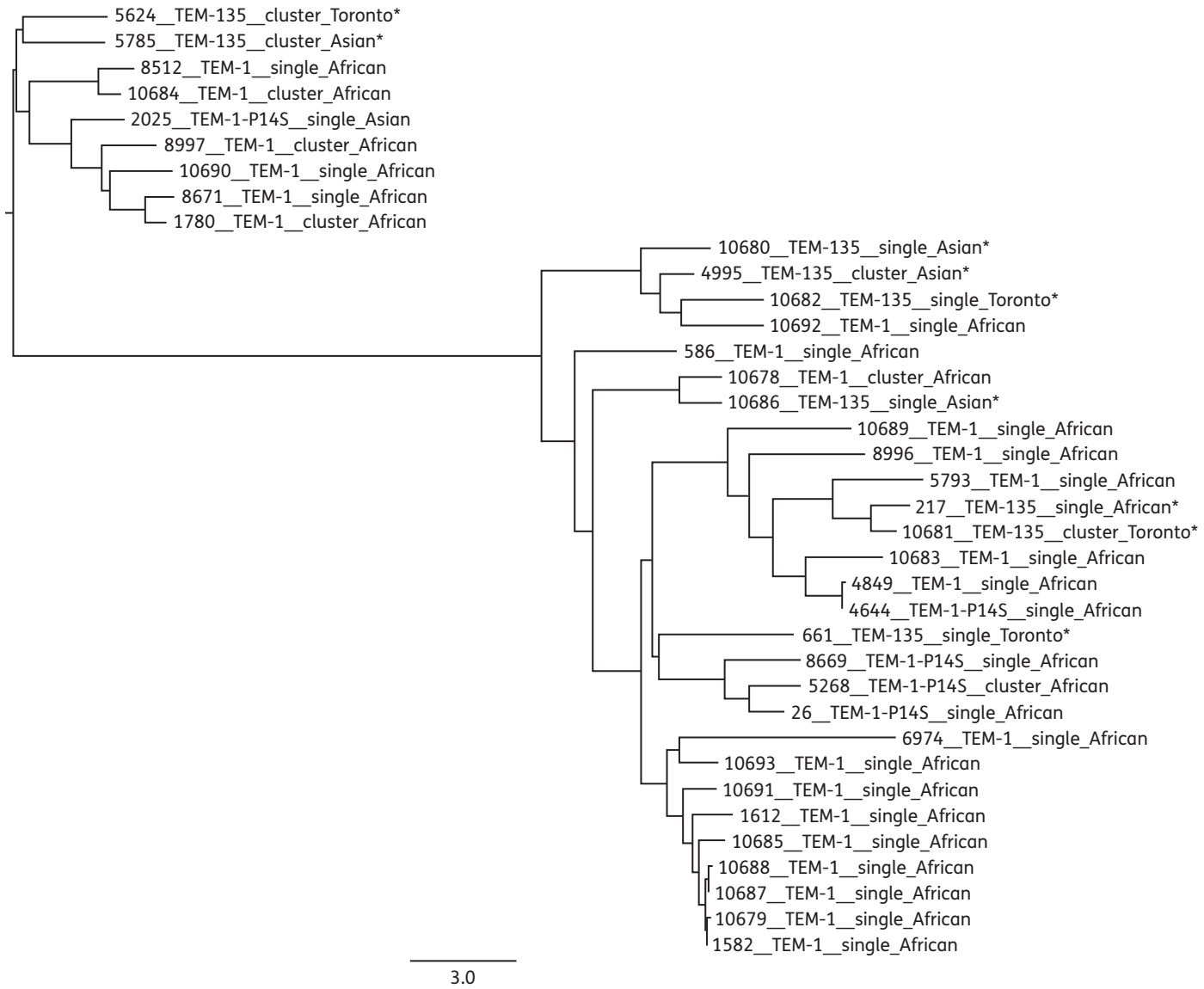

Figure 2. Phylogram of concatenated *porB* and *tbpB* alleles (880 bp) from PPNG isolates from England and Wales (2012). Node labels: NG-MAST ST_TEM enzyme_single ST or cluster (at least two isolates)_plasmid type. Asterisks show NG-MAST STs from TEM-135 isolates.

stability.[27–30] The recent global PPNG study[10] revealed that the degradation of ampicillin by TEM-1 and TEM-135 was indistinguishable using a relatively simple hydrolysis assay, indicating no disadvantage of the M182T substitution.

TEM-135 has been circulating in gonococci since at least 1984 and so pre-dated the increased use of extended-spectrum cephalosporins for gonorrhoea treatment.[10] Increased stability conferred by M182T far from the active site, in the hinge region between the two β-lactamase domains,[21] may have allowed this variant to become fixed in the population. Alternatively, M182T may simply be a natural polymorphism that confers no disadvantage and so is maintained within the gonococcal population as an efficient penicillin resistance mechanism. Our single-year study does not indicate when TEM-135 emerged in the gonococcal population in England and Wales. Further sequencing of plasmids with TEM-135 and genomes from historical isolates may help in answering this question, as well as to determine whether TEM-135 has spread among the different β-lactamase plasmid types by horizontal transfer or has occurred *in situ* through SNPs in TEM-1. The three TEM variants detected in this collection of gonococci from England and Wales, TEM-135, TEM-1 and TEM-1 (P14S), have all been detected elsewhere.[10]

Even though extended-spectrum cephalosporin use may not have selected for TEM-135 in *N. gonorrhoeae*,[10] the fact remains that just a specific SNP near the β-lactamase active site in this already stable enzyme could result in TEM-135 evolving into an ESBL. Currently, there are three ESBLs that differ from TEM-135 by only one amino acid substitution (TEM-20, TEM-106 and TEM-126).[31] Even though resistance and decreased susceptibility to extended-spectrum cephalosporins in gonococci has, to date, been chromosomally mediated,[2] it is not unreasonable to foresee the emergence of further mutations in the TEM-1 or TEM-135

enzymes in *N. gonorrhoeae*, selected by increasing extended-spectrum cephalosporin dosages. The potential emergence of an ESBL in the TEM-135 gonococcal population, in the UK and additional countries, is of additional concern as these isolates already display high-level ciprofloxacin and tetracycline resistance. This situation therefore requires monitoring as the emergence and spread of an ESBL in *N. gonorrhoeae* would render the last remaining treatment option for gonorrhoea, i.e. ceftriaxone, useless and would be a public health emergency.

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Author contributions

M. J. C. and N. W. designed, initiated and coordinated the study. M. J. C., V. G. and N. Q. performed the laboratory analyses. M. J. C., M. U. and N. W. analysed and interpreted the data and wrote the first draft of the paper. All authors read, commented and approved the final manuscript.

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