International Journal of Antimicrobial Agents 54 (2019) 361–366

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

High prevalence of TEM-135 expression from the Asian plasmid in penicillinase-producing *Neisseria gonorrhoeae* from Hangzhou, China

Jing Yan[a], Jianglin Zhang[a], Stijn van der Veen[a,b,c,*]

[a] Department of Microbiology and Parasitology, School of Medicine, Zhejiang University, Hangzhou, China
[b] Department of Dermatology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China
[c] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

ARTICLE INFO

Article history:
Received 8 January 2019
Accepted 10 June 2019

Editor: Po-Ren Hsueh

Keywords:
Gonococcus
Penicillinase-producing *Neisseria gonorrhoeae*
PPNG
β-Lactamase
bla genes
Penicillin

ABSTRACT

Penicillinase-producing *Neisseria gonorrhoeae* (PPNG) expressing the TEM β-lactamase variant TEM-135 are a global public-health concern as this variant requires only a single amino acid substitution to develop into an extended-spectrum β-lactamase. The aim of this study was to investigate the epidemiology of $bla_{\text{TEM-135}}$ in 505 *N. gonorrhoeae* isolates from Hangzhou, China, during the periods 2011–2012 and 2015–2017. Investigation by nitrocefin test and mismatch amplification PCR showed that 41.0% (207/505) of the isolates were PPNG, of which 37.2% (77/207) contained the $bla_{\text{TEM-135}}$ gene. Further PCR-based plasmid typing showed that $bla_{\text{TEM-135}}$ was predominantly expressed from the Asian plasmid (94%). PPNG isolates consisted of three major clusters, namely Asian plasmid/$bla_{\text{TEM-135}}$ (34.8%), Asian plasmid/$bla_{\text{TEM-1}}$ (32.4%) and African plasmid/$bla_{\text{TEM-1}}$ (28.0%), which showed significant differences in penicillin minimum inhibitory concentrations (MICs) determined by the agar dilution method. Representative isolates were investigated by quantitative real-time PCR (plasmid copy number and bla_{TEM} gene expression), western blot analysis (TEM levels and TEM stability) and in vivo β-lactamase activity assays to elucidate the cause of the observed differences in penicillin MIC. Overall, isolates of the Asian plasmid/$bla_{\text{TEM-135}}$ cluster showed the highest β-lactamase activity, which was explained by higher bla_{TEM} gene expression (Asian versus African plasmid) and higher TEM stability (TEM-135 versus TEM-1). In conclusion, the $bla_{\text{TEM-135}}$ gene is commonly present on the Asian plasmid in PPNG isolates from Hangzhou. The PPNG isolate cluster containing the Asian plasmid and $bla_{\text{TEM-135}}$ showed the highest penicillin MICs, which might explain its abundance in the Hangzhou population.

1. Introduction

The development of multidrug resistance in the bacterial pathogen *Neisseria gonorrhoeae* is a major public-health concern and novel antimicrobial treatments are urgently required [1]. Currently, the last available first-line monotherapy for treatment of gonococcal infections is the cephalosporin ceftriaxone. However, resistance levels to ceftriaxone are rising and confirmed treatment failures with ceftriaxone have been reported in several countries (summarised in [2]). Resistance to ceftriaxone is generally the result of multiple mutations in *penA*, encoding penicillin-binding protein 2 (PBP2), the target of ceftriaxone [3]. In addition, limiting ceftriaxone influx by *porB* mutations and increasing efflux by mutations that induce expression of the MtrCDE multidrug efflux pump are commonly observed [3]. Thus far, extended-spectrum β-lactamases (ESBLs), which are able to hydrolyse the cyclic amide bond in the β-lactam ring of cephalosporins and render them inactive, have not been encountered in *N. gonorrhoeae*. However, penicillinase-producing *N. gonorrhoeae* (PPNG) isolates are common [4]. PPNG isolates display high-level resistance to penicillin but their β-lactamase is not active against cephalosporins.

Until now, all investigated PPNG isolates contained a TEM-type β-lactamase, which is encoded by a bla_{TEM} gene carried on a group of closely related plasmids, most frequently the Asian, African and Toronto/Rio types [4,5]. Traditionally, PPNG isolates expressed the TEM-1 variant, but more recently the TEM-135 variant has also been encountered in many countries [4,6–11]. TEM-135 differs from TEM-1 by an M182T mutation that is the result of a thymine to cytosine transition at position 539. This M182T mutation improves the stability of the β-lactamase and allows otherwise deleterious mutations in the active site to arise that could

* Corresponding author. Present address: Zhejiang University School of Medicine, 866 Yuhangtang Road, Hangzhou 310058, P.R. China. Tel.: +86 571 8820 6684; fax: +86 571 8820 8022.
E-mail address: stijnvanderveen@zju.edu.cn (S. van der Veen).

https://doi.org/10.1016/j.ijantimicag.2019.06.012
0924-8579/

evolve it into an ESBL [12]. Several TEM-type ESBLs have already been described that differ from TEM-135 by one or two amino acid substitutions and that allow more flexibility in the active site and confer the ability to hydrolyse cephalosporins [12]. Although these cephalosporin-hydrolysing TEM-135 derivatives have not yet been encountered in *N. gonorrhoeae*, the emergence and spread of PPNG isolates expressing TEM-135 is a major concern because it might signify the future development of *N. gonorrhoeae* expressing ESBLs, which would render the currently last available anti-gonococcal monotherapy inactive.

In China, PPNG isolates are highly abundant but only a few studies have further characterised these isolates for their plasmid types and bla_{TEM} genes [6,13,14]. Therefore, the current study aimed to characterise the plasmid type and bla_{TEM} genes of PPNG isolates identified in a collection of isolates from Hangzhou covering the periods 2011–2012 and 2015–2017 previously characterised for antimicrobial susceptibility [15,16].

2. Materials and methods

2.1. Neisseria gonorrhoeae isolates, penicillinase-producing N. gonorrhoeae testing, plasmid typing and identification of $bla_{TEM-135}$

This study included 505 *N. gonorrhoeae* isolates obtained from seven hospitals in the Hangzhou area of China during the periods 2011–2012 and 2015–2017. These isolates have been characterised for antimicrobial susceptibility and sequence type (ST) in our previous studies [15,16]. PPNG isolates were identified using nitrocefin solution (Sigma-Aldrich Co., St Louis, MO). A phylogenetic tree of PPNG isolates was constructed in MEGA7 with concatenated *porB* and *tbpB* genes [15]. Plasmid types as well as bla_{TEM}, *penA*, *porB* and *mtrR* genes were identified by PCR as described previously [7,17]. Complete plasmids for 15 representative isolates were sequenced by Sanger sequencing and were deposited in GenBank under the accession nos. **MK973072**–**MK973086**.

2.2. Analysis of plasmid copy number and bla_{TEM} gene expression by quantitative real-time PCR (qPCR)

DNA was extracted from 1 mL of bacterial suspension [optical density at 600 nm (OD_{600}) = 1] using an EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, Shanghai, China) according to the manufacturer's protocols. Total RNA isolation and cDNA synthesis was performed as described previously [18]. DNA and cDNA samples were used in qPCR analysis [18] using primers Q-blaTEM-F (AGACTGGATGGAGGCGGATAAAG), Q-blaTEM-R (TAGATAACTACGATACGGGAGGGC), Q-recA-F (GCCGTCGCCCAATGCCAG), Q-recA-R (CGGACGAGCGTGTCGCAGAT), Q-16S-F (AGCCGTAACACAGGTGCTGCAT) and Q-16S-R (GCCCAACCGAATGATGGCAACT). Plasmid copy numbers were determined as the ratio between bla_{TEM} signal (plasmid) and *recA* (genomic, one copy) or 16S rDNA (genomic, four copies) signal. Relative bla_{TEM} gene expression levels were determined after normalisation for *recA* and 16S rRNA expression levels.

2.3. Expression and purification of recombinant TEM-1 and RecA, and generation of antisera

The complete open-reading frames of bla_{TEM-1} and *recA* were expressed in *Escherichia coli* BL21(DE3) from a pEASY®-E2 vector (TransGen Biotech). Expression and purification of TEM-1 and RecA was performed by nickel affinity with a HisTrap FF column (GE Healthcare, Uppsala, Sweden) as described previously [19]. TEM-1 and RecA (50 μg/dose) were formulated with Freund's complete or incomplete adjuvant (Sigma-Aldrich Co.) according to the manufacturer's recommendations. Two groups of five female 6-week-old BALB/c mice (SLAC, Shanghai, China) were immunised subcutaneously on Day 0 (Freund's complete adjuvant), Day 21 (Freund's incomplete adjuvant) and Day 35 (Freund's incomplete adjuvant) and sera were collected on Day 49. Sera for each group were pooled, aliquoted and stored at −80 °C until use.

2.4. Protein stability and western blotting

Overnight-grown bacteria were suspended in 25 mL of GC broth (Oxoid Ltd., Basingstoke, UK) containing 1% (v/v) Vitox (Oxoid Ltd.). Cultures were incubated at 37 °C and 200 rpm until $OD_{600} = 0.5$ was reached and then samples were taken to determine protein expression levels by western blot analysis. For determination of protein stability, chloramphenicol (7.5 mg/L) (Inalco SpA, Milan, Italy) was added to inhibit protein expression and samples were collected after 0, 0.5, 1, 2, 3 and 4 h of incubation. Protein levels were detected with TEM-1 or RecA antisera (1:500) and horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Abbkine Scientific Co. Ltd., Wuhan, China). Expression levels of TEM were quantified with ImageJ (National Institutes of Health, Bethesda, MD) and were normalised to the levels of RecA.

2.5. TEM activity assay

Overnight-grown bacteria were suspended in phosphate-buffered saline to a concentration of ca. 10^8 CFU/mL. Bacterial aliquots of 150 μL were transferred to 96-well plates and the reaction was started with the addition of 2.5 μg nitrocefin (5 μL). The reaction was followed by measuring the OD_{490} every minute. A reference line was generated with a dilution series of nitrocefin and recombinant purified TEM-1 to correlate the ΔOD_{490} in time to nmol nitrocefin conversion.

3. Results

3.1. Prevalence of penicillinase-producing Neisseria gonorrhoeae, plasmid type and $bla_{TEM-135}$ gene

Of the 505 isolates, 207 (41.0%) were PPNG, including 41/65 (63%) in 2011, 31/61 (51%) in 2012, 45/127 (35%) in 2015, 52/145 (36%) in 2016 and 38/107 (36%) in 2017. Therefore, the prevalence of PPNG over the last 3 years (35–36%) remained stable. The majority of the 207 PPNG isolates contained the Asian plasmid [32 in 2011, 24 in 2012, 31 in 2015, 28 in 2016 and 24 in 2017; total, 139/207 (67.1%)] or the African plasmid (9 in 2011, 5 in 2012, 13 in 2015, 21 in 2016 and 14 in 2017; total, 62/207 (30.0%)], whilst only 3 isolates contained the Toronto/Rio plasmid and another 3 isolates contained other rare plasmid types. Further analysis of the bla_{TEM} genes showed that 77 (37.2%) of the PPNG isolates contained $bla_{TEM-135}$ (22 in 2011, 14 in 2012, 12 in 2015, 11 in 2016 and 18 in 2017) and 130 isolates contained bla_{TEM-1} (19 in 2011, 17 in 2012, 33 in 2015, 41 in 2016 and 20 in 2017). Importantly, 94% (72/77) of the $bla_{TEM-135}$ positive PPNG isolates contained the Asian plasmid, which is therefore the main plasmid responsible for the distribution of the $bla_{TEM-135}$ gene in the Hangzhou area.

3.2. Molecular epidemiology analysis of penicillinase-producing Neisseria gonorrhoeae isolates

The 207 PPNG isolates were assigned to 139 different STs, with ST1927 (7 isolates), ST1866 (6 isolates), ST3102 (6 isolates) and ST2318 (5 isolates) being the most prevalent. Further analysis of STs associated with the different plasmids showed that the Asian plasmid-containing isolates belonged to 103 different STs, with ST1866 (6 isolates), ST3102 (6 isolates), ST1893 (4 isolates) and ST436 (4 isolates) being most commonly observed. The African plasmid was present in isolates belonging to 45 different STs, with

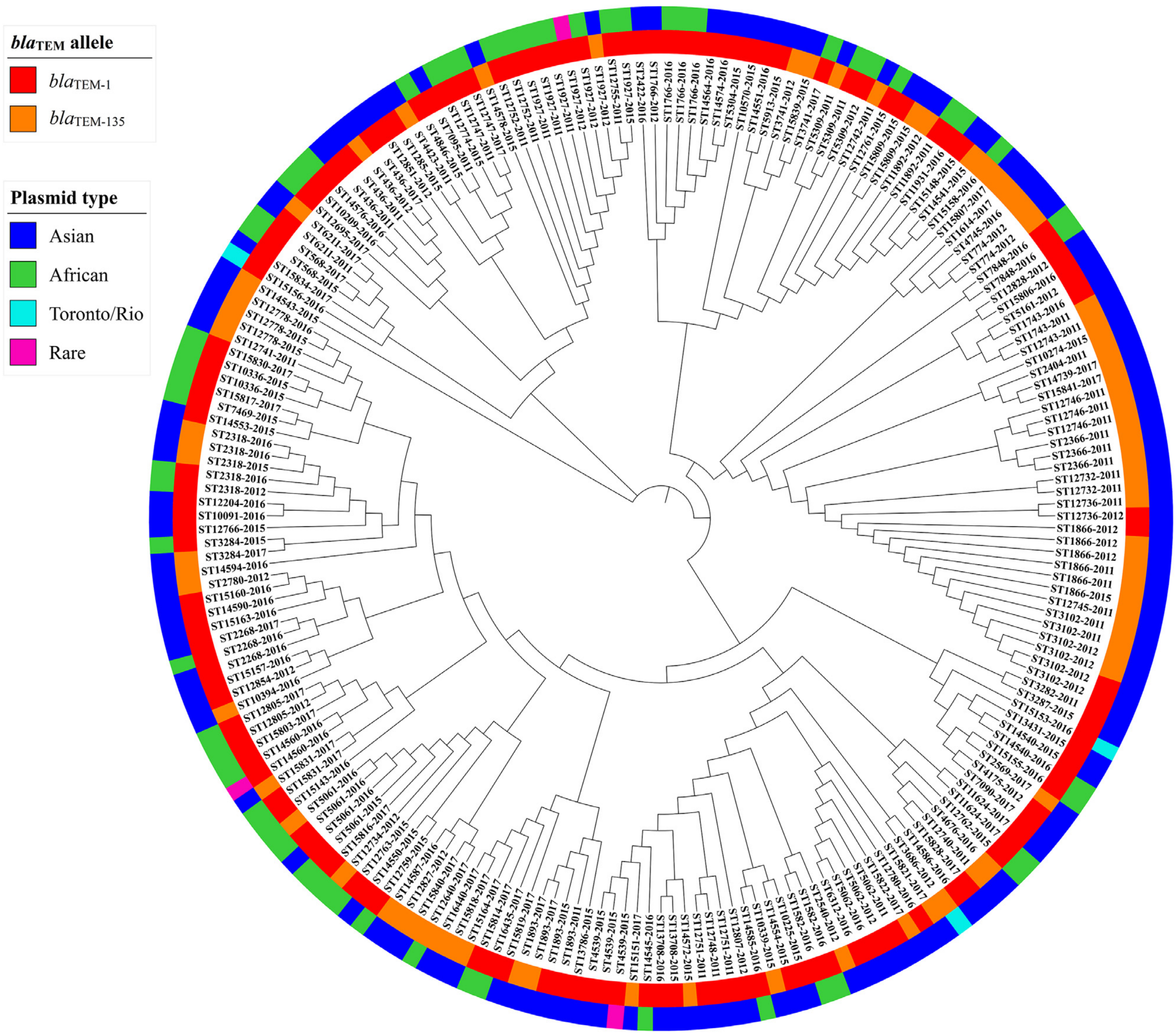

Fig. 1. Genetic relationship between penicillinase-producing *Neisseria gonorrhoeae* isolates containing different plasmid types and bla_{TEM} genes. Concatenated *porB* and *tbpB* alleles were used to construct the phylogenetic tree in MEGA7. Sequence types (STs) are shown and the plasmid type and bla_{TEM} gene are indicated by colour coding.

ST1927 (5 isolates) being most common. Similarly, the $bla_{TEM-135}$ gene was present in 59 different STs and was most commonly observed in isolates belonging ST1866 (5 isolates) and ST3102 (4 isolates). To obtain greater insight into the genetic relationship between PPNG isolates containing the $bla_{TEM-135}$ gene, a phylogenetic tree was constructed and plasmid types and bla_{TEM} genes were indicated with colour coding (Fig. 1). The $bla_{TEM-135}$ gene was observed in PPNG isolates spread throughout the tree but, importantly, several clusters of $bla_{TEM-135}$-containing isolates were also observed, including one large cluster ranging from ST3102 and ST1866 to ST1614. Almost all isolates in this cluster contained the *tbpB* allele 33 and related *por* alleles. Therefore, this large clonally related cluster of isolates containing $bla_{TEM-135}$ might pose a particular threat for the development of ESBLs. Importantly, isolates belonging to ST1866 and ST3102 are also high-level azithromycin-resistant [15,16].

3.3. Penicillin susceptibility of penicillinase-producing *Neisseria gonorrhoeae* isolates

The PPNG isolates can be divided into three major clusters, namely isolates containing the Asian plasmid and $bla_{TEM-135}$ ($n = 72$), isolates containing the Asian plasmid and bla_{TEM-1} ($n = 67$) and isolates containing the African plasmid and bla_{TEM-1} ($n = 58$). These clusters of isolates were further analysed for penicillin susceptibility. Penicillin minimum inhibitory concentrations (MICs) for the Asian plasmid-containing isolates were significantly higher compared with the MICs of the African plasmid-containing isolates (Fig. 2a). This difference was plasmid-dependent since a significant difference was still observed when only the bla_{TEM-1}-containing isolates were analysed for both plasmid types (Fig. 2b). Importantly, comparison of penicillin MICs between the bla_{TEM-1}- and $bla_{TEM-135}$-containing Asian plasmid clusters showed that penicillin MICs were significantly higher for the $bla_{TEM-135}$ cluster (Fig. 2c). Therefore, it appears that both the plasmid type and the bla_{TEM} gene have an impact on the resistance levels to penicillin.

3.4. Impact of plasmid type and bla_{TEM} gene on expression and activity levels

To investigate the differences in penicillin MICs between the isolates containing the Asian and African plasmid and the bla_{TEM-1} and $bla_{TEM-135}$ genes, five isolates with diverse penicillin MICs were selected from each of the three major PPNG

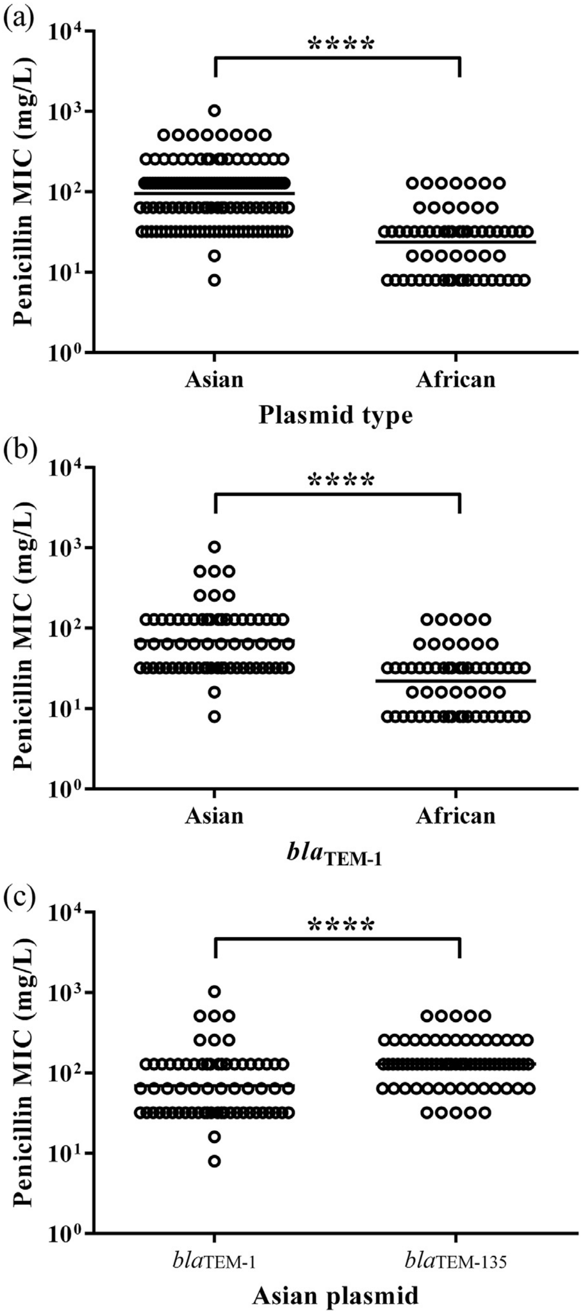

Fig. 2. Penicillin minimum inhibitory concentrations (MICs) of penicillinase-producing *Neisseria gonorrhoeae* isolates according to plasmid type and/or bla_{TEM} gene. The penicillin MIC for individual isolates is represented by an open circle. The horizontal line indicates the geometric mean MIC for each group. (a) Distribution of penicillin MICs according to plasmid type. (b) Distribution of penicillin MICs according to plasmid type for strains containing $bla_{\text{TEM-1}}$ only. (c) Distribution of penicillin MICs according to bla_{TEM} gene for strains containing the Asian plasmid only. Significant differences were identified by the Mann–Whitney *U*-test using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). **** $P < 0.0001$.

clusters: Asian/$bla_{\text{TEM-135}}$, 512 mg/L (*penA*12.008, *mtrR*1, *porB*8), 256 mg/L (*penA*2.002, *mtrR*19, *porB*11), 128 mg/L (*penA*43.001, *mtrR*1, *porB*4), 64 mg/L (*penA*43.002, *mtrR*22, *porB*13) and 16 mg/L (*penA*21.001, *mtrR*19, *porB*11); Asian/$bla_{\text{TEM-1}}$, 512 mg/L (*penA*12.008, *mtrR*1, *porB*11), 256 mg/L (*penA*2.002, *mtrR*1, *porB*8), 128 mg/L (*penA*13.001, *mtrR*1, *porB*9), 64 mg/L (*penA*43.001, *mtrR*1, *porB*4) and 32 mg/L (*penA*5.002, *mtrR*38, *porB*8); and African/$bla_{\text{TEM-1}}$, 128 mg/L (*penA*43.001, *mtrR*16, *porB*4), 64 mg/L (*penA*103.001, *mtrR*1, *porB*8), 32 mg/L (*penA*2.008, *mtrR*25, *porB*13), 16 mg/L (*penA*19.001, *mtrR*47, *porB*13) and 8 mg/L (*penA*19.001, *mtrR*38, *porB*12). All 15 isolates contained polymorphisms in *mtrR* and *porB* that have previously been associated with β-lactam resistance, and none of the isolates contained a mosaic *penA* allele. Whole-plasmid sequence analysis for the 15 isolates showed >99% sequence identity with the previously published Asian and African plasmids [5] and, except for the M182T mutation, no differences were observed in bla_{TEM} and its promoter region. These isolates were further analysed by qPCR for plasmid copy number and bla_{TEM} gene expression. All isolates contained approximately four copies of the Asian or African plasmid (Fig. 3a), however gene expression in the African plasmid-containing isolates was significantly lower compared with the Asian plasmid-containing isolates (Fig. 3b). This reduced gene expression was also reflected in the TEM protein levels, since the African plasmid-containing isolates showed significantly lower TEM levels compared with the Asian plasmid-containing isolates (Fig. 3c). Interestingly, within the Asian plasmid-containing isolates, isolates containing $bla_{\text{TEM-135}}$ showed significantly higher TEM levels compared with isolates containing $bla_{\text{TEM-1}}$. This difference in protein levels could be explained by differences in protein stability between TEM-1 and TEM-135. Therefore, the relative stability/degradation of TEM was investigated in the bacteria after switching off protein synthesis, which confirmed that the relative stability of TEM-1 was lower compared with TEM-135 (Fig. 3d). Finally, these differences in bla_{TEM} gene expression and TEM levels impacted the in vivo β-lactamase activity, since the Asian/$bla_{\text{TEM-135}}$ isolates displayed the highest activity and the African/$bla_{\text{TEM-1}}$ isolates the lowest (Fig. 3e). Therefore, the observed differences in penicillin MICs between the three major PPNG clusters were explained by differences in bla_{TEM} gene expression and TEM stability, which impacted the total protein levels and β-lactamase activity.

4. Discussion

The emergence and spread of PPNG isolates expressing TEM-135 is a major concern because TEM-135 contains a stabilising mutation that possibly allows it to evolve into an ESBL [12]. In the current study, the $bla_{\text{TEM-135}}$ gene was encountered in 37.2% of the PPNG isolates and was predominantly present on the Asian plasmid, which is similar to observations from the UK [11] but is in contrast with several other recent studies from Thailand [7] and Argentina [10] as well as a global study [4] which showed that $bla_{\text{TEM-135}}$ was mostly associated with the Toronto/Rio plasmid type. In Australia [9] and France [20], the $bla_{\text{TEM-135}}$ gene was also commonly present in the Toronto/Rio plasmid type, but over one-half of the Asian plasmids also contained the $bla_{\text{TEM-135}}$ gene in these studies. In China, the Asian plasmid has already been observed in several other studies as the most common plasmid type in PPNG isolates [13,14]. However, information on the distribution of the $bla_{\text{TEM-135}}$ gene in China is very limited. In Nanjing, 58% of PPNG isolates from 2007 and 2012 contained the $bla_{\text{TEM-135}}$ gene [6]. Importantly, three overlapping $bla_{\text{TEM-135}}$-containing STs were observed in the Nanjing study, namely ST1866, ST2318 and ST4745. Specifically isolates of ST1866, which were part of the largest cluster of genetically related $bla_{\text{TEM-135}}$-containing isolates, appear to be spreading in Eastern and Southeastern China and isolates of this ST commonly display high-level azithromycin resistance [13,15]. Therefore, these isolates might be of particular concern for countries currently recommending ceftriaxone plus azithromycin dual-therapy as first-line treatment.

The PPNG isolates from the current study could be divided in three major clusters based on plasmid type and bla_{TEM} gene. Interestingly, the results demonstrated that penicillin MICs were different between the three major PPNG clusters, which was the result of differences in bla_{TEM} gene expression levels between the

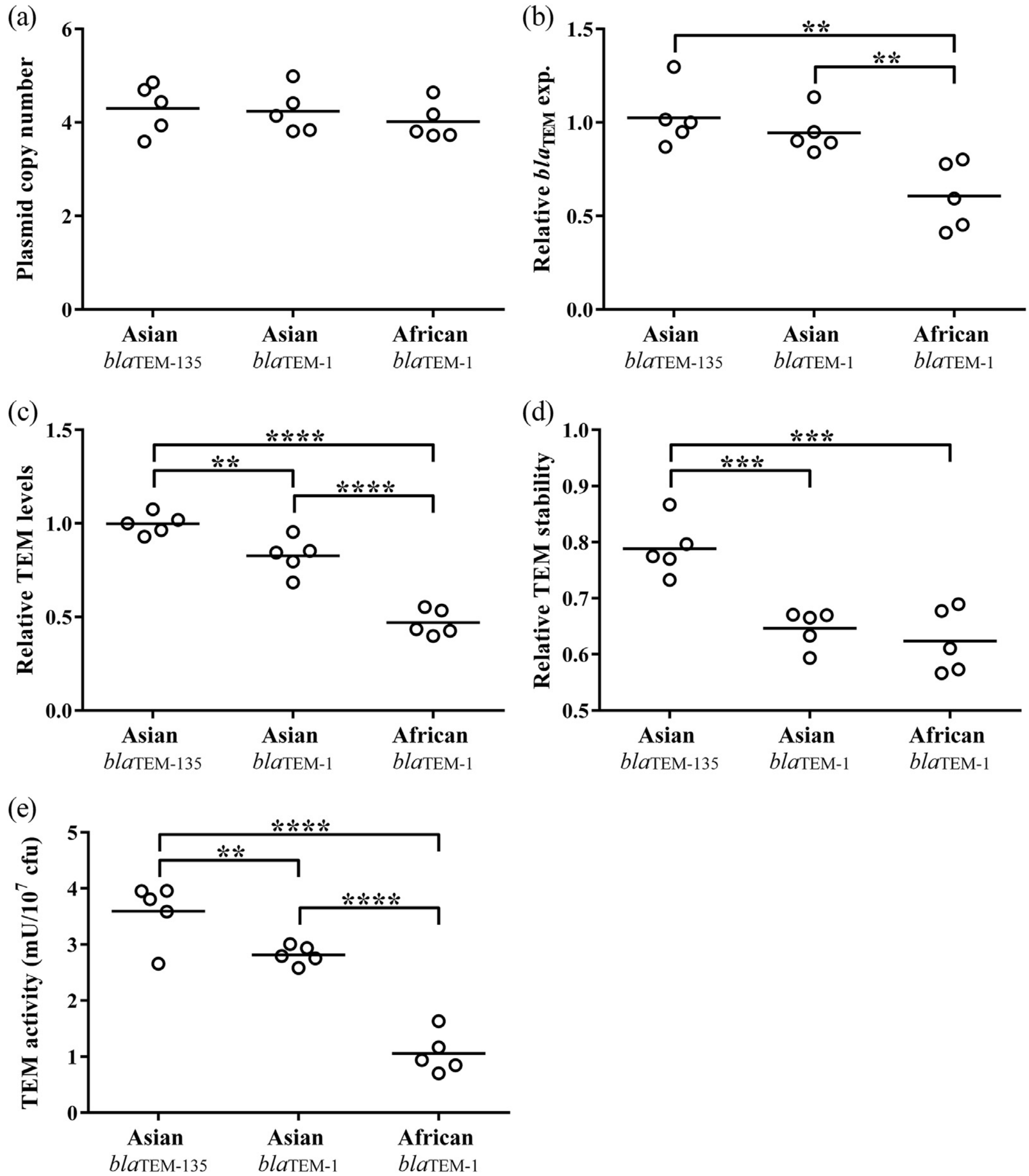

Fig. 3. Characterisation of β-lactamase expression, stability and activity levels in isolates belonging to the three major penicillinase-producing *Neisseria gonorrhoeae* clusters. Five isolates with diverse penicillin minimum inhibitory concentrations (MICs) were selected from the Asian/$bla_{\text{TEM-135}}$, Asian/$bla_{\text{TEM-1}}$ and African/$bla_{\text{TEM-1}}$ clusters. All graphs represent data from three biological repeats and the average results for individual strains are represented by open circles. (a) Plasmid copy number analysed by quantitative real-time PCR (qPCR). (b) Relative gene expression of bla_{TEM} analysed by qPCR. (c) Relative TEM levels quantified by western blot analysis and normalised to RecA levels. (d) Relative TEM stability quantified by western blot analysis and normalised to RecA stability levels. (e) Maximum β-lactamase activity quantified by nitrocefin conversion. One unit (U) of β-lactamase activity is defined as the amount of enzyme that converts 1.0 nmol of nitrocefin per minute at pH 7.0 at 25 °C. Significant differences were identified by one-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Asian and African plasmid types as well as differences in TEM stability between TEM-1 and TEM-135, affecting their total intracellular TEM levels and activity. Although these differences have not been reported before, a previous study from Japan showed that all Asian plasmid-containing isolates ($n = 4$) displayed a penicillin MIC > 64 mg/L, whilst the African plasmid-containing isolates ($n = 5$) displayed MICs of 16–64 mg/L [8]. Admittedly, the isolate numbers analysed in that study were too low to draw any sound conclusions on differences in penicillin MICs between Asian or African plasmid-containing isolates. The complete Asian and African plasmids have previously been sequenced [5], but bla_{TEM} promoter regions from both plasmids are identical and could not explain the observed differences in bla_{TEM} gene expression.

5. Conclusion

Here we showed that the $bla_{\text{TEM-135}}$ gene was highly abundant in PPNG isolates from the Hangzhou area and was commonly present on the Asian plasmid. In addition, isolates containing the Asian plasmid and $bla_{\text{TEM-135}}$ showed the highest penicillin MICs, which could be beneficial for its dissemination into the broader public given that penicillin is still one of the most commonly used antibiotics globally and *N. gonorrhoeae* is frequently carried asymptomatically. Dissemination of $bla_{\text{TEM-135}}$ is worrisome since a single additional single nucleotide polymorphism could give it the ability to encode an ESBL, which would render the last remaining first-line treatment of gonorrhoea ineffective.

Declaration of Competing Interest

None declared.

Funding

This work was supported by the Zhejiang Provincial Natural Science Foundation of China [grant no. LR16H190001]. The funder had no role in study design, data collection and interpretation, writing of the manuscript, or the decision to submit the manuscript for publication.

Ethical approval

The animal studies and procedures were approved by the Zhejiang University Animal Care and Use Committee [project license no. ZJU2015-032-01]. The procedures followed the guidelines of Administration of Affairs Concerning Experimental Animals of the People's Republic of China, which adhere to the principles of the Declaration of Helsinki.

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