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Molecular Analyses of TEM Genes and Their Corresponding Penicillinase-Producing *Neisseria gonorrhoeae* Isolates in Bangkok, Thailand

Shu-ichi Nakayama,[a] Chanwit Tribuddharat,[b] Sasiprapa Prombhul,[b] Ken Shimuta,[a] Somporn Srifuengfung,[b] Magnus Unemo,[c] and Makoto Ohnishi[a]

Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan[a]; Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand[b]; and Swedish Reference Laboratory for Pathogenic Neisseria, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden[c]

***Neisseria gonorrhoeae* is a major public health problem globally, especially because the bacterium has developed resistance to most antimicrobials introduced for first-line treatment of gonorrhea. In the present study, 96 *N. gonorrhoeae* isolates with high-level resistance to penicillin from 121 clinical isolates in Thailand were examined to investigate changes related to their plasmid-mediated penicillin resistance and their molecular epidemiological relationships. A β-lactamase (TEM) gene variant, $bla_{\text{TEM-135}}$, that may be a precursor in the transitional stage of a traditional $bla_{\text{TEM-1}}$ gene into an extended-spectrum β-lactamase (ESBL), possibly causing high resistance to all extended-spectrum cephalosporins in *N. gonorrhoeae*, was identified. Clonal analysis using multilocus sequence typing (MLST) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) revealed the existence of a sexual network among patients from Japan and Thailand. Molecular analysis of the $bla_{\text{TEM-135}}$ gene showed that the emergence of this allele might not be a rare genetic event and that the allele has evolved in different plasmid backgrounds, which results possibly indicate that it is selected due to antimicrobial pressure. The presence of the $bla_{\text{TEM-135}}$ allele in the penicillinase-producing *N. gonorrhoeae* population may call for monitoring for the possible emergence of ESBL-producing *N. gonorrhoeae* in the future. This study identified a bla_{TEM} variant ($bla_{\text{TEM-135}}$) that is a possible intermediate precursor for an ESBL, which warrants international awareness.**

Neisseria gonorrhoeae is the causative agent of gonorrhea, which is the second most prevalent bacterial sexually transmitted infection globally. During recent decades, *N. gonorrhoeae* has rapidly developed resistance to most classes of antimicrobials used for treatment of gonorrhea (4, 6, 17, 18, 20). Penicillinase-producing *N. gonorrhoeae* (PPNG), with plasmid-mediated high-level resistance to penicillin, was first reported in 1976 (1, 14) and has since been disseminated worldwide (2). The first gonococcal strain with high-level clinical resistance to ceftriaxone, which is the last remaining option for first-line gonorrhea treatment, was recently found in Japan and completely characterized (9, 11). However, the resistance to ceftriaxone was chromosomally mediated, and no extended-spectrum β-lactamase (ESBL) has yet been identified in *N. gonorrhoeae*. If an ESBL did emerge in *N. gonorrhoeae* and spread internationally, gonorrhea would become an extremely serious public health problem.

PPNG strains are rare in Japan, but these strains have remained highly prevalent in several other countries in Asia (19) and worldwide (20). Penicillin is still also used as the first-line drug in, e.g., some Pacific island countries and the northern part of Australia, because of maintained efficacy in the settings and its low cost.

Although the β-lactamase (TEM) gene of authentic PPNG is the $bla_{\text{TEM-1}}$ allele, a recently isolated PPNG in Thailand possessed the $bla_{\text{TEM-135}}$ allele, which differs from the $bla_{\text{TEM-1}}$ allele with one single nucleotide polymorphism (SNP) at position 539, resulting in a single amino acid substitution, M182T (16). However, the prevalence and characteristics of TEM-135 strains worldwide are unknown and seem critical to study, especially in countries where PPNG strains are highly prevalent. Furthermore, the knowledge regarding the genetic relationships of PPNG strains, their TEM genes, and plasmids carrying β-lactamase is highly limited.

Therefore, in the present study, PPNG isolates cultured from 2005 to 2007 in Thailand, which has a relatively high prevalence of PPNG, were investigated. To detect $bla_{\text{TEM-135}}$ in the PPNG strains, a simple and rapid mismatch amplification mutation assay (MAMA) PCR method (3) was developed and successfully used. To reveal the population structure of the PPNG isolates, molecular epidemiological typing by means of multilocus sequence typing (MLST) (5), *porB* gene sequencing, and *N. gonorrhoeae* multiantigen sequence typing NG-MAST (7) were used to compare the detected TEM-135 strains with the TEM-1 strains.

MATERIALS AND METHODS

Bacterial isolates. *N. gonorrhoeae* isolates were collected from Siriraj Hospital, Bangkok, Thailand. Among 121 isolates collected during 2005 to 2007, based on resistance to penicillin and a positive nitrocefin test, a total of 96 PPNG isolates were detected and analyzed (see the supplemental material). These isolates were systematically collected in a previous research project (16).

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Address correspondence to Makato Ohnishi, ohnishi7@nih.go.jp.
S. Nakayama and C. Tribuddharat contributed equally to this work.
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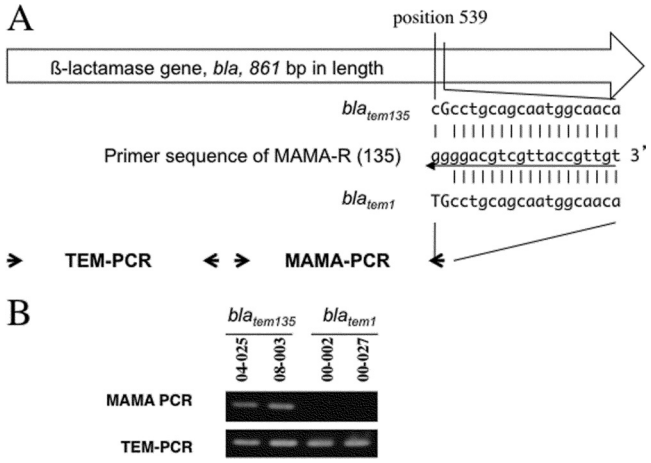

FIG 1 MAMA-PCR for $bla_{\text{TEM-135}}$ detection. (A) The TEM PCR primer set (TEM-F and TEM-R), which can amplify a 231-bp amplicon from $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$, and the MAMA-PCR primer set, specific for $bla_{\text{TEM-135}}$ (MAMA-F and MAMA-R), are shown schematically with arrows. The sequence of primer MAMA-R (middle) and the corresponding regions from bla_{tem135} (top) and bla_{tem1} (bottom) are also shown. (B) The PCR results for the Japanese penicillinase-producing *N. gonorrhoeae* (PPNG) TEM-135 and PPNG TEM-1 isolates, which were used as controls in all PCRs, are presented.

DNA isolation. To obtain genomic DNA, isolates were suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and boiled for 10 min. After removing cell debris by centrifugation, the supernatant was used directly as template DNA in the PCR.

PCR identification of bla_{TEM} gene. The MAMA-PCR to detect sequence polymorphism between the $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$ alleles focused on nucleotide position 539 of the bla_{TEM} gene (Fig. 1A). A conserved forward primer (MAMA-F, 5′-GCATCTTACGGATGGCATGAC-3′) and a $bla_{\text{TEM-135}}$ allele-specific polymorphism detection primer (MAMA-R, 5′-TGTTGCCATTGCTGCAGG**GG**-3′) were designed (Table 1). The $bla_{\text{TEM-135}}$ allele-specific primer carries a specific nucleotide, G (bold and underlined), at the 3′ end. Furthermore, to enhance the 3′ end mismatch effect, an additional nucleotide alteration of G, rather than C (bold), at the second nucleotide from the 3′ end of the primer was introduced. Thus, the $bla_{\text{TEM-135}}$ allele-specific primer contained two mismatched bases at the 3′ end relative to the sequence of $bla_{\text{TEM-1}}$ (Fig. 1A). In brief, the 10-μl-volume PCR master mix contained diluted template DNA, 0.8 μl of 2.5 mM deoxynucleoside triphosphate (dNTP) mixture (final concentration, 200 μM each), 0.25 μl each of 10 μM MAMA-F and MAMA-R primers (final concentration, 250 nM each), and 0.25 units of the DNA polymerase Takara Ex *Taq* (Takara Bio Co., Kyoto, Japan). The parameters of the PCRs were as follows: incubation for 2 min at 96°C followed by 25 cycles of 10 s at 96°C, 10 s at 56°C, and 30 s at 72°C and then final extension for 2 min at 72°C. The previously described *N. gonorrhoeae* strains NGON 00-002 and NGON 00-027 (containing $bla_{\text{TEM-1}}$) and NGON 04-025 and NGON 08-003 (containing $bla_{\text{TEM-135}}$) (10) were used as controls in all PCRs (Fig. 1). The universal TEM PCR was done as described above except that the PCR master mix contained TEM-F and TEM-R primers (Table 1). To confirm TEM alleles, we sequenced PCR-amplified products of the whole bla_{TEM} coding region using the primer set bla-F and bla-R as described previously (10).

Molecular epidemiological characterization. Molecular epidemiological characterization by means of MLST (5), *porB* gene sequencing, and NG-MAST was performed as described previously (7). The type of plasmid carrying the β-lactamase (TEM) gene was determined by a multiplex PCR method developed by Palmer et al. (12). Neighbor-joining trees with *por* and *tbpB* nucleotide sequences were generated by using MEGA4.

Drawing of minimum spanning tree. Based on the MLST data, a minimum spanning tree was generated by using BioNumerics (version 5.1; Applied Math), using the categorical coefficient of similarity and the priority rule of the highest number of single-locus variants as parameters. No hypothetical sequence or reported sequences other than those identified in the present study were included in the calculation.

RESULTS AND DISCUSSION

Development and use of the MAMA-PCR for detection of $bla_{\text{TEM-135}}$. To differentiate the $bla_{\text{TEM-135}}$ allele from the $bla_{\text{TEM-1}}$ allele in PPNG strains, by detection of the SNP at position 539, a MAMA-PCR (detecting only $bla_{\text{TEM-135}}$) was successfully developed and was used together with a TEM PCR (detecting both $bla_{\text{TEM-135}}$ and $bla_{\text{TEM-1}}$) (Fig. 1).

Nine of the 96 PPNG isolates from Thailand were positive in both the MAMA-PCR and TEM PCR, suggesting that these isolates possessed the $bla_{\text{TEM-135}}$ allele. Sequencing analysis of the full-length PCR products from the *bla* gene confirmed that these nine isolates (9.4%) indeed contained the $bla_{\text{TEM-135}}$ allele, and the remaining 87 isolates (90.6%) possessed $bla_{\text{TEM-1}}$.

Genetic relationships of PPNG TEM-1 and PPNG TEM-135 isolates. In order to examine the genetic relationships of PPNG isolates containing TEM-1 and TEM-135, MLST was carried out. Twenty-three MLST STs were identified among the 96 PPNG isolates, 17 STs among the TEM-1 isolates and 6 among the TEM-135 isolates. Among the 17 MLST STs identified among the TEM-1 isolates, ST1588 was the most prevalent (55 out of the 87 TEM-1 isolates, 63.2%) (Table 2). A minimum spanning tree analysis showed that most of the other STs in TEM-1 isolates were closely related to ST1588, with few exceptions (Fig. 2). Accordingly, 83 out of the 87 (95.4%) TEM-1 isolates belonged to a large cluster comprising 15 STs and centered around ST1588 (cluster A) (Fig. 2 and Table 2). The remaining four TEM-1 isolates were assigned ST8782 ($n = 2$) and ST8775 ($n = 2$), which formed an additional smaller cluster (cluster B) (Fig. 2 and Table 2).

Six different MLST STs were found in the nine TEM-135 isolates (Fig. 2 and Table 2). ST8778 was the most common ($n = 4$, 44.4%), and the other five STs were singletons. All these TEM-135 isolates, with the exception of the singleton ST7822 (isolate Thai_026) that was placed in the TEM-1 cluster A, belonged to the same separate cluster (cluster C) (Fig. 2 and Table 2). Taken together, Thailand PPNG TEM-1 and PPNG TEM-135 strains seem to belong to distinct clonal groupings with different genetic backgrounds, and also, TEM-135 strains have emerged from multiple independent origins.

Plasmid typing. Plasmid typing has been used as another classification method for PPNG surveillance. We also performed plasmid typing and investigated relationships with the results of MLST and the specific alleles of the *bla* genes $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$.

As shown in Table 2, the Africa-type β-lactamase plasmid was the predominant type (79 of 96 isolates, 82.3%) in the isolates

TABLE 1 Primers used in the MAMA-PCR for detection of $bla_{\text{TEM-135}}$ and the TEM-PCR for detection of both $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$

Primer	Primer sequence (5′ to 3′)	Position
MAMA-F	GCATCTTACGGATGGCATGAC	327-347
MAMA-R[a]	TGTTGCCATTGCTGCAGGGG	558-539
TEM-F	GTCGCCCTTATTCCCTTTTTTG	22-43
TEM-R	TAGTGTATGCGGCGACCGAG	284-268

[a] Binds only $bla_{\text{TEM-135}}$.

TABLE 2 MLST sequence type and plasmid type of PPNG isolates cultured in Thailand in 2005 to 2007[a]

No. of isolates	MLST		No. of isolates with plasmid type[c]:		
	ST	Cluster[b]	Africa	Asia	Toronto/Rio
55	1588	A	53	1	1
5	8780	A	5		
4	1903	A	4		
4	8774	A	4		
2	1584	A	2		
2	1921	A	2		
2	8779	A	2		
1	7827	A	1		
1	8145	A	1		
1	8776	A	1		
1	8783	A	1		
1	8777	A	1		
2	7823	A		2	
1	1600	A		1	
1	7822	A		1 (1)[d]	
1	8781	A		1	
2	8775	B		2	
2	8782	B	2		
4	8778	C			4 (4)
1	1582	C			1 (1)
1	8136	C			1 (1)
1	8143	C			1 (1)
1	8784	C			1 (1)
96		TOTAL	79	8 (1)	9 (8)

[a] MLST, multilocus sequencing typing (5); PPNG, penicillinase-producing *Neisseria gonorrhoeae*.
[b] Clusters were defined by the minimum spanning tree in Fig. 2.
[c] Plasmid typing was determined by a multiplex PCR (12).
[d] The number of PPNG TEM-135 isolates is shown in parentheses.

analyzed in the present study. Asia- and Toronto/Rio-type β-lactamase plasmids were found in only eight and nine isolates, respectively. Recently, a new type of the β-lactamase plasmid (Johannesburg plasmid) was reported by Muller et al. (8). If the Johannesburg-type plasmid had existed in our isolates, it would have generated a 450-bp amplicon with the BL1 and BL3 primers in our multiplex PCR system. However, we did not find any isolates containing this plasmid. Notable, all TEM-135 isolates, except Thai_026 (MLST ST7822) which had an Asia-type plasmid, carried the Toronto/Rio-type plasmid. As described above, Thai_026 (MLST ST7822) was the only isolate that belonged to cluster A formed by the TEM-1 isolates. Thus, the plasmid typing supported separation of this isolate from the other TEM-135 isolates, which further supports the hypothesis that TEM-135 strains have emerged from multiple independent origins. There was no TEM-135 isolate with the Africa-type plasmid. On the other hand, although all three plasmid types were found among the TEM-1 isolates, the Africa-type plasmid was the most abundant among the TEM-1 isolates (79 out of 87 isolates, 90.8%). Thus, this plasmid typing, again, implied a genetic difference of TEM-135 and TEM-1 strains. The most abundant MLST ST in TEM-1 isolates, ST1588, was strongly related to the Africa-type plasmid (53 out of 55 isolates, 96.4%). The remaining two MLST ST1588 isolates carried Toronto/Rio- and Asia-type plasmids, respectively. In total, the Africa-type plasmid was also abundant in other MLSTs, although both of the MLST ST8775 isolates had the Asia-type plasmid. Other isolates with the Asia-type plasmid were limited to MLST ST8781, MLST ST7822 (single TEM-135 isolate), MLST ST7823, and MLST ST1600 (Table 2). Interestingly, three of these MLST ST (except MLST ST1600) were linked and formed a stem in the left part of the minimum spanning tree (Fig. 2).

NG-MAST analysis. To thoroughly evaluate the genetic diversity and relatedness of the TEM-135 isolates, all PPNG isolates were also analyzed using a substantially more discriminative typing method, NG-MAST (7). The 96 PPNG isolates were divided into 58 NG-MAST STs. Each NG-MAST ST is shown in the supplemental material.

Among the four TEM-135 isolates assigned to MLST ST8788, three belonged to NG-MAST ST5134 (*porB3109* and *tbpB98*; Fig. 3A), indicating clonal dissemination. Also, four of the additional TEM-135 isolates contained highly similar *porB* alleles (Thai_098, Thai_045, Thai_003, and Thai_032) (Fig. 3B). This similarity and, accordingly, the clustering were further supported by analyzing the *tbpB* alleles of all the TEM-135 isolates (Fig. 3C). Accordingly, the NG-MAST supported the conclusion that seven of the nine TEM-135 isolates had originated from a common ancestor. Both the remaining TEM-135 isolates (Thai_026 and Thai_073) were genetically separated from this cluster by the NG-MAST (Fig. 3). This was also in full concordance with the results of the MLST and plasmid typing (Table 2 and Fig. 2).

Comparison of the Thai isolates with previously characterized Japanese PPNG isolates. Using several molecular typing methods, we tried to identify any spread of PPNG between Thailand and Japan and found the possible spread of only one TEM-1

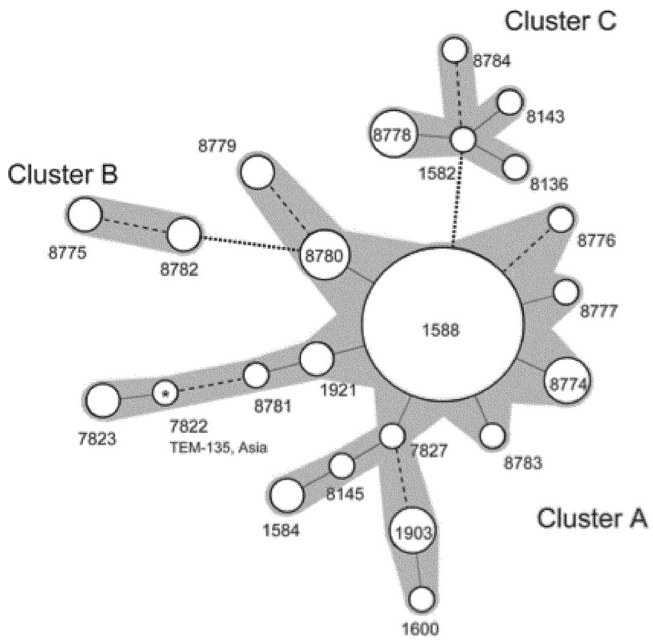

FIG 2 Minimum spanning tree analysis of multilocus sequence typing (MLST) STs observed in penicillinase-producing *N. gonorrhoeae* (PPNG) isolates cultured in Thailand in 2005 to 2007. Numbers beside the circles denote ST. Solid lines, dashed lines, and dotted lines show the interrelationship of "single-locus variant," "double-locus variant," and "triple-locus variant," respectively. The types directly or indirectly connected through single- or double-locus-variant relationships were judged to form one cluster. Each cluster is shaded gray. Sizes of circles reflect the numbers of isolates belonging to each type (for details, see text and tables). The only PPNG TEM-135 isolate belonging to cluster A (ST7822) is marked with an asterisk, and its plasmid type is given.

A

Strain	MLST	*por*	*tbpB*	NG-MAST
Thai_022, _078, _127	8778	3109	98	5134
Thai_003	8778	2809	1084	5138
Thai_045	8143	1830	1088	5132
Thai_098	8784	832	98	1292
Thai_032	1582	53	21	147
Thai_026	7822	3110	186	5135
Thai_073	8136	90	58	211

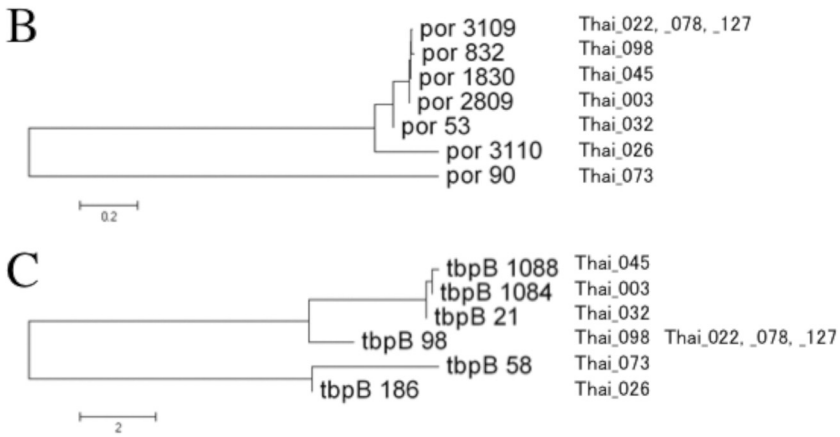

FIG 3 Molecular characterization of penicillinase-producing *N. gonorrhoeae* PPNG TEM-135 isolates. (A) Sequence types revealed by multilocus sequence typing (MLST) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) are shown, along with *por* and *tbpB* alleles. (B and C) Neighbor-joining clustering showing similarity of *por* alleles (B) and *tbpB* alleles (C) from PPNG TEM-135 isolates.

clone. Accordingly, the previously characterized Japanese isolates NGON 08-041 and NGON 08-046 (10) and Thai_036 and Thai_093 were all, in the present study, assigned to MLST ST1584 and NG-MAST ST1478 and carried the Africa-type plasmid with $bla_{\text{TEM-1}}$. Despite some similarities in the MLST STs supporting, e.g., a cluster of isolates with the TEM-135 Toronto/Rio-type plasmid, no clear evidence to support international spread of any TEM-135 strains was found.

It is well-known that the β-lactamase plasmid can also easily be transferred between different *N. gonorrhoeae* strains. As the number of analyzed isolates in the present study was relatively low and they were cultured in restricted regions, Thailand (Bangkok) and Japan (Tokyo), more extensive international studies are crucial to reveal the origin and the evolutionary pathway of the TEM-135 strains, as well as the possible existence of PPNG with other TEM alleles.

Possible motive force of emergence of TEM-135. Still, the reasons and mechanisms for the emergence and dissemination of PPNG TEM-135 strains are unknown. The $bla_{\text{TEM-135}}$ allele was first found in *Salmonella enterica* serovar Typhimurium (13), and there are no major differences in the MICs of any β-lactam antimicrobials between $bla_{\text{TEM-135}}$ and $bla_{\text{TEM-1}}$ allele-possessing isolates. The $bla_{\text{TEM-135}}$ allele has now been found in two different types of β-lactamase plasmid in PPNG, which are known to originally carry the $bla_{\text{TEM-1}}$ allele. This fact indicates that $bla_{\text{TEM-135}}$ emerged independently in *N. gonorrhoeae* and was not acquired due to, for example, a transformational event. However, due to the similar MICs of β-lactam antimicrobials in PPNG TEM-1 and TEM-135 isolates, other factor(s) than β-lactam antimicrobial selective pressure must be the selective force in the emergence of $bla_{\text{TEM-135}}$. One possibility might be a pressure by other antibiotic(s) than penicillins. If so, we could expect some different patterns of resistance or rate of resistance to nonpenicillin antibiotics between TEM-1 and TEM-135 isolates. However, we did not observe any significant difference in those, at least when comparing susceptibility and resistance to ceftriaxone, ciprofloxacin, and tetracycline (data not shown). Another possibility is that this selective force may be an enhanced stability of the β-lactamase enzyme, which the TEM-135-specific amino acid substitution (M182T) is considered to establish (15, 21). Usually, this amino acid substitution is found in extended-spectrum TEM-type β-lactamase, as the second substitution. Since an amino acid substitution close to the active site of β-lactamase, which results in an increased MIC of cephalosporins, tends to decrease the stability of the enzyme, the M182T substitution may play a role as a stabilizer. In this context, the M182T in $bla_{\text{TEM-135}}$ in PPNG might be a prerequisite to allow the subsequent substitutions, which could extend the antimicrobial resistance spectrum of the enzyme, like several TEM-type β-lactamases found in other bacteria, e.g., TEM-20 carriers.

Necessity of monitoring TEM-135 PPNG. In conclusion, an emergence of ESBL-producing *N. gonorrhoeae* would be highly threatening to public health, because this would also be resistant to ceftriaxone, which is the first-line and last remaining option for treatment of *N. gonorrhoeae* infection in many countries world-

wide. Recently, the first *N. gonorrhoeae* strain with chromosomally mediated high-level resistance to ceftriaxone was isolated in Japan (9, 11). Although this strain was not PPNG, i.e., it had a *penA*-dependent resistance mechanism, this calls for a substantially strengthened monitoring of ceftriaxone-resistant *N. gonorrhoeae* infection and gonorrhea treatment failures, including consideration of possible emergence of ESBL-producing *N. gonorrhoeae* isolates.

In Thailand, about 10% of PPNG had TEM-135, a possible direct precursor of an ESBL. However, the prevalence and characteristics of TEM-135 strains and possible strains containing other TEM variants worldwide is unknown. This seems crucial to investigate in larger, international studies, including studies of recent geographically, phenotypically, and genetically diverse PPNG.

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