Short Communication

Gonococcal Antimicrobial Susceptibility and the Prevalence of \( \text{bla}_{\text{TEM}-1} \) and \( \text{bla}_{\text{TEM}-135} \) Genes in \textit{Neisseria gonorrhoeae} Isolates from Thailand

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\textbf{SUMMARY:} We studied the antimicrobial susceptibility and prevalence of the \( \text{bla}_{\text{TEM}-1} \) and \( \text{bla}_{\text{TEM}-135} \) genes in \textit{Neisseria gonorrhoeae} isolates obtained in Thailand. The isolates were tested using the disk diffusion method, and 100% of 370 isolates were found susceptible to cefixime, ceftriaxone, ceftazidime, cefepime, spectinomycin, and azithromycin. Some of the isolates were resistant to penicillin (85.7%), ciprofloxacin (88.0%), ofloxacin (97.4%), or tetracycline (89.1%). Penicillinase-producing \textit{N. gonorrhoeae} accounted for 83.8% of isolates, with 70.0% of these further identified as penicillinase-producing plus tetracycline resistant \textit{N. gonorrhoeae}. Penicillin, tetracycline, and ciprofloxacin are not recommended for treatment because of the high prevalence (89.7%) of multidrug resistant gonococci. A study of genes controlling enzyme of beta-lactamase production (\( \text{bla}_{\text{TEM}-1} \) and \( \text{bla}_{\text{TEM}-135} \)) was performed using mismatch amplification mutation assay PCR method and DNA sequencing. Beta-lactamase positive \textit{N. gonorrhoeae} carried \( \text{bla}_{\text{TEM}-1} \) (69.6%) and \( \text{bla}_{\text{TEM}-135} \) (30.4%), indicating that there is a significant increase and spread of \( \text{bla}_{\text{TEM}-135} \) among gonococci in Thailand.

Each year, gonococci infect more than 106 million people globally, with approximately 700,000 cases in the USA alone (1). Gonococci cause disease in peoples of all ages and, with less frequency, is associated with severe complications e.g., pelvic inflammatory disease, ectopic pregnancy, infertility in women and men, and blindness in congenitally-infected infants (2). Third-generation cephalosporins i.e., cefixime (oral) and ceftriaxone (injectable), are the last remaining drugs of choice for these infections. However, cefixime resistance has been reported in Japan (3), France (4), Austria, England, and Norway (2), and these resistant isolates have spread. At present, cefixime is not recommended for the treatment of gonococcal infection in many countries. In contrast, a study from 2005 to 2007 showed that gonococci in Thailand were not resistant to cefixime (5). The World Health Organization and U.S. Centers for Disease Control and Prevention (CDC) have issued a new guideline, dual therapy (ceftriaxone plus azithromycin) for the treatment of gonococcal infection. However, there are reports of azithromycin-resistant gonococci with reduced susceptibility to ceftriaxone in many countries, e.g., Canada (6), as well as the emergence of multidrug resistance or reduced susceptibility to ceftriaxone in France (4), Switzerland (7), Spain (2), and Japan (8).

Penicillinase-producing \textit{N. gonorrhoeae} (PPNG) which are prevalent worldwide, contains a plasmid carrying a gene to produce beta-lactamase, which hydrolyzes the cyclic amide bond of the beta-lactam ring in penicillin. A plasmid encoding the TEM-1 enzyme has spread in gonococci. Interestingly, the first report of PPNG from Thailand showed gonococci carrying \( \text{bla}_{\text{TEM}-135} \) gene with the amino acid at position 182 of the \( \text{bla}_{\text{TEM}-1} \) gene changed from methionine to threonine. The sequences of this gene were 100% identical to that found in \textit{Salmonella enterica} (5). Furthermore, when the \( \text{bla}_{\text{TEM}-135} \) gene acquires an additional mutation, these bacteria may express extended-spectrum beta-lactamase (9). This study aimed to determine the current status of drug resistance and rates of \( \text{bla}_{\text{TEM}-1} \) and \( \text{bla}_{\text{TEM}-135} \) genes in \textit{N. gonorrhoeae} in Thailand.

A total of 370 isolates were obtained from patients at the Siriraj Hospital (340 patients) and Bangrak Hospital (30 patients) from January 2008 to December 2014. Multiple isolates from different sites in the same patient were included only once in the study. Gonococci were identified by the Vitek 2 system using the NH ID card (Biomerieux, Hazelwood, MO, USA). Isolates were stored at \(-80^\circ\text{C}\) in 5% trypticase soy broth plus 20% v/v glycerol until use. The susceptibility of the isolates to penicillin, cefixime, ceftazidime, ceftriaxone, cefotaxime, ciprofloxacin, ofloxacin, tetracycline, and azithromycin was determined using the disk diffusion method (10). \textit{N. gonorrhoeae} ATCC 49226 was used as a control. Because there is no guideline on susceptibility to azithromycin by the Clinical and Laboratory Standard Institutes (CLSI), we used the interpretation of the CDC \textit{Neisseria} Reference Laboratory, indicating that in which gonococci were susceptible with an inhibition zone diameter of \( \geq 30 \text{ mm} \) (11).

To identify PPNG, we performed a beta-lactamase
were randomly selected for DNA sequencing using PCR (MAMA-PCR) (9). The negative control for the β-lactamase gene was N. gonorrhoeae ATCC 49226. Previously identified gonococcal isolates numbers 5 (9) and 22 (5) were used as positive controls for the β-lactamase and β-lactamase-135 genes, respectively. Purified PCR products were randomly selected for DNA sequencing using PCR-specific primers as previously described (9) to confirm the presence of β-lactamase and β-lactamase-135 genes (First Base Laboratory, Selangor, Malaysia). The sequence of PCR products were used for a homology search by nucleotide blast (blastn) and protein blast (blastx) of the GenBank database for best matches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

The age range of patients whose samples were included in this study was 2–71 years with a mean ± standard deviation (SD) of 22.88 ± 11.59 years. The woman: man ratio was 1.34:1. Most patients were in age range, 15–19 years (32.23%), followed by 20–24 years (16.80%), 10–14 years (14.05%), and 25–29 years (9.92%), which accounted for 73.00% of patients. No data on patients’ ages were available for 7 isolates. Specimens obtained included pus from the urethra (17.57%, 65 isolates), cervix (34.05%, 126 isolates), vagina (1.35%, 5 isolates), eyes (6.22%, 38 isolates), synovial fluid (joint fluid; 6.95%, 25 isolates), and others (0.81%, 3 isolates).

Results of antimicrobial susceptibility testing (Table 1) showed that 100% of gonococcal isolates were susceptible to cefixime, ceftriaxone, ceftaxime, ceftazidime, cefepime, spectinomycin, and azithromycin. Some isolates were resistant to penicillin (85.7%), ciprofloxacin (88.0%), ofloxacin (97.4%), or tetracycline (89.1%). Our results agree with those of other studies that have shown gonococci that remain susceptible to tetracycline, e.g., Nepal (12) and Hungary (13).

In this study, the MICs of ceftriaxone, cefixime, and azithromycin were determined by E-test (AB Biodisk, Solna, Sweden) which showed MICs ranging 0.002–0.032, 0.016–0.023, and 0.006–0.25 μg/ml, respectively.

PPNG accounted for 83.8% of isolates with 70.0% of these further identified as penicillinase-producing plus tetracycline-resistant N. gonorrhoeae. Penicillin, tetracycline, and ciprofloxacin (Table 2) are not recommended for treatment because of the high prevalence (89.7%) of these multidrug-resistant (resistant to at least 3 antimicrobial classes) gonococci.

The prevalence of β-lactamase-1 (69.6%) and β-lactamase-135 (30.4%) in 280 PPNG isolates was determined (Table 3). The β-lactamase-135 gene was initially identified in Bangkok (5), and, in a 2005–2007 survey, 10% of gonococci were found to contain β-lactamase-135 (9). A high prevalence of β-lactamase-135 was found in China (58%) in 2007–2012 (14), and β-lactamase-135 isolates have also been detected in Japan, Australia, and 15 other countries (15). Of 42 β-lactamase-135 isolates collected in Argentina from 2008 to 2012, 2 isolate were identified as β-lactamase-135 with a novel mutation and determined a new TEM family β-lactamase-220 (15).

The full TEM gene (868 bp) of 6 randomly selected isolates (3 β-lactamase-1 and 3 β-lactamase-135 based on MAMA-PCR) was sequenced. We confirmed that 3 isolates were β-lactamase-1, and 3 isolates were β-lactamase-135 type. The sequences of β-lactamase-1 were 100% identical to that of the E. coli strain MROB16 beta-lactamase (β-lactamase-1) gene, GenBank accession no. KM593703. The sequences of β-lactamase-135 were 100% identical to that of N. gonorrhoeae isolate 22 beta-lactamase-TEM-135 (β-lactamase-135) gene, GenBank accession no. GQ896333. However, the TEM genes of the other 82 β-lactamase-135 and 192 β-lactamase-1

Table 1. Antimicrobial susceptibility of gonococci using disk diffusion method

| Antimicrobial agent | n | Susceptible | Intermediate | Resistant |
|---------------------|---|-------------|--------------|-----------|
| penicillin          | 350 | 1.7 | 12.6 | 85.7 |
| cefixime            | 289 | 100 | — | — |
| ceftriaxone         | 350 | 100 | — | — |
| ceftaxime           | 50  | 100 | — | — |
| ceftazidime         | 300 | 100 | — | — |
| cefepime            | 81  | 100 | — | — |
| ciprofloxacin       | 349 | 2.6 | 9.4 | 88.0 |
| ofloxacin           | 301 | 1.7 | 3.6 | 94.7 |
| tetracycline        | 350 | 6.9 | 4.0 | 89.1 |
| spectinomycin       | 30  | 100 | — | — |
| azithromycin        | 281 | 100 | — | — |

Table 2. Penicillin, tetracycline, and ciprofloxacin resistance in gonococci

| Antimicrobial agent | n | % |
|---------------------|---|---|
| (PEN + TET + CIP)-S | 0 | 0.0 |
| PEN-nonS            | 1 | 0.3 |
| TET-nonS            | 0 | 0.0 |
| CIP-nonS            | 2 | 0.6 |
| (PEN + TET)-nonS    | 8 | 2.3 |
| (PEN + CIP)-nonS    | 21| 6.0 |
| (TET + CIP)-nonS    | 4 | 1.1 |
| (PEN + TET + CIP)-nonS | 313 | 89.7 |

Total 349 | 100 |

PEN, penicillin; TET, tetracycline; CIP, ciprofloxacin; S, susceptible; nonS, intermediate + resistant.

Table 3. Distribution of β-lactamase genes between 2008 and 2014

| Yr | n | %β-lactamase-1 (n) | %β-lactamase-135 (n) |
|----|---|-------------------|---------------------|
| 2008 | 23 | 78.3 (18) | 21.7 (5) |
| 2009 | 24 | 83.3 (20) | 16.7 (4) |
| 2010 | 48 | 70.8 (34) | 29.2 (14) |
| 2011 | 44 | 70.5 (31) | 29.5 (13) |
| 2012 | 53 | 75.5 (40) | 24.5 (13) |
| 2013 | 31 | 48.4 (15) | 51.6 (16) |
| 2014 | 57 | 64.9 (37) | 35.1 (20) |

Total 280 | 69.6 (195) | 30.4 (85) |
were not completely sequenced (Table 3).

The diameter of the inhibition zones (mean ± SD, in mm) of all antimicrobial agents were compared between all bla<sub>TEM-1</sub> and bla<sub>TEM-135</sub> isolates. The results indicated that bla<sub>TEM-135</sub> isolates had a significantly smaller zone of inhibition for penicillin only: penicillin (15.2 ± 5.3 for bla<sub>TEM-1</sub>, 7.4 ± 1.9 for bla<sub>TEM-135</sub>); cefixime (41.9 ± 4.9, 41.6 ± 5.4); ceftriaxone (46.8 ± 4.3, 47.0 ± 2.9); cefotaxime (47.3 ± 4.4, 48.6 ± 3.4); ceftazidime (41.3 ± 4.5, 40.0 ± 4.7); cefepime (47.5 ± 6.2, 47.7 ± 3.7); ciprofloxacin (19.2 ± 7.8, 16.1 ± 4.5); ofloxacin (15.8 ± 6.4, 13.4 ± 3.9); tetracycline (14.3 ± 6.6, 14.6 ± 8.1); spectinomycin (27.2 ± 2.7, 28.0 ± 5.4); and azithromycin (38.6 ± 3.7, 37.4 ± 3.7). Even though inhibition zone diameters do not completely correlate with MIC values, our data suggest that the penicillin MICs of isolates harboring bla<sub>TEM-135</sub> may be higher than of those harboring bla<sub>TEM-1</sub>. On the other hand, according to conventional thought, MICs of bla<sub>TEM-135</sub> are indistinguishable from that of bla<sub>TEM-1</sub> (15). The results for the MIC showed complete correlation with our data for diameter of the inhibition zone. Therefore, our data may suggest that the penicillin MICs of isolates harboring bla<sub>TEM-135</sub> are not completely sequenced (Table 3).

In conclusion, continuous monitoring of antimicrobial resistance is necessary to limit the spread of resistant strains. This report shows a significant increase in bla<sub>TEM-135</sub> (10% (9) to 30.4%; [p value < 0.01]) indicating the spread of bla<sub>TEM-135</sub> among gonococci in Thailand.

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Conflict of interest None to declare.

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