# *In vitro* activity of ertapenem against *Neisseria gonorrhoeae* clinical isolates with decreased susceptibility or resistance to extended spectrum cephalosporins in Nanjing, China (2013-2019)

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**Running Title:** Susceptibility of *Neisseria gonorrhoeae* to ertapenem

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**ABSTRACT**

**Objective:** *Neisseria gonorrhoeae* isolates collected in Nanjing, China, that possessed decreased susceptibility (or resistance) to extended spectrum cephalosporins (ESCs), were examined for susceptibility to ertapenem and their sequence types determined.

**Methods:** Ceftriaxone and cefixime minimum inhibitory concentrations (MICs) ≥ 0.125 mg/L and ≥ 0.25 mg/L, respectively, were first determined in 259 strains isolated between 2013 and 2019 and then MICs of ertapenem were measured using the antimicrobial gradient epsilometer test (Etest). Genetic determinants of ESC resistance and multi-antigen sequence typing (NG-MAST) were also determined to analyze associations with ertapenem susceptibility.

**Results:** All isolates displayed ertapenem MICs between 0.006 mg/L-0.38 mg/L; the overall $MIC_{50}$ and $MIC_{90}$ were 0.032 mg/L and 0.125 mg/L. 44 (17.0%) isolates displayed ertapenem MICs of ≥ 0.125 mg/L; 10 (3.9%) had MICs ≥ 0.25 mg/L. The proportion of isolates with ertapenem MICs ≥ 0.125 mg/L increased from 4.0% in 2013, to 20.0% in 2019 ($\chi^2$= 24.144, P<0.001; Chi square test for linear trend). The *penA* mosaic allele was present in a significantly higher proportion of isolates with ertapenem MICs ≥ 0.125 mg/L compared to isolates with MICs ≤ 0.094 mg/L) (97.7% vs. 34.9%, respectively; $\chi^2$=58.158, P<0.001). ST5308 was the most prevalent

NG-MAST type (8.5%); ST5308 was also significantly more common among isolates with ertapenem MICs ≥ 0.125 mg/L vs. isolates with MICs ≤ 0.094mg/L (22.7% and 5.6% respectively; $\chi^2$=13.815, P=0.001).

**Conclusions:** Ertapenem may be effective therapy for gonococcal isolates with decreased susceptibility or resistance to ESCs and isolates with identifiable genetic resistance determinants.

**Keywords:** *Neisseria gonorrhoeae*, ertapenem, ESCs, resistance

## INTRODUCTION

Gonorrhea is the second most common bacterial sexually transmitted infection and a major global public health problem. The World Health Organization (WHO) estimated that 87 million new cases occurred worldwide in adults aged 15-49, in 2016 (1). In China, the incidence of gonorrhea increased by 36.03% (7.05 to 9.59 cases per 100,000 population) from 2014 to 2018 (2). Treatment of gonorrhea is challenging because *N. gonorrhoeae* has developed resistance to most antimicrobials (AMR) that have been used for therapy, including sulfonamides, penicillins, tetracyclines, fluoroquinolones, early-generation and, rarely, extended-spectrum cephalosporins (ESCs) (3-7).

Currently, ceftriaxone monotherapy or dual therapy with ceftriaxone or cefixime plus azithromycin is recommended as first-line treatment of uncomplicated gonorrhea in most countries (8-10). Unfortunately, strains resistant to ESCs and azithromycin are emerging globally (9, 11) and treatment failures with currently-recommended dual

67 therapies have been reported (12, 13). Thus, currently recommended treatments are
68 unlikely to continue to be effective long-term; exploring novel or repurposed
69 antimicrobials will be essential for control of gonorrhea (7).
70
71 Ertapenem is a parenteral carbapenem, effective against gram-negative bacteria that
72 may, otherwise, be resistant to cephalosporins. Similar to other β-lactams, ertapenem
73 inhibits cell wall synthesis by binding to and inhibiting penicillin-binding proteins
74 (PBPs) (14). It is well tolerated, effective and has a safety profile comparable to that
75 of ceftriaxone (15, 16). Ertapenem has been used successfully to treat *N. gonorrhoeae*
76 with both high-level azithromycin and ceftriaxone resistance (13) and may be an
77 effective treatment option for gonorrhea, particularly infections caused by strains
78 resistant to extended spectrum cephalosporins (ESCs).
79
80 No specific genetic determinants of ertapenem resistance or carbapenemases,
81 generally, have been identified in *N. gonorrhoeae*; however, there may be overlap
82 with resistant mechanisms exhibited by other ESCs (17). Mechanisms of resistance
83 against ESCs can result from amino acid changes caused by nucleotide mutations in:
84 *penA* (encoding penicillin-binding protein 2, PBP2); *mtrR* (encoding the multiple
85 transfer resistance repressor, MtrR); *penB* (encoding porin PorB) and *ponA* (encoding
86 penicillin-binding protein 1, PBP1) in *N. gonorrhoeae* (3, 18-21).
87
88 The major aim of the present study was to examine *in vitro* activity of ertapenem,

against *N. gonorrhoeae* isolates with decreased susceptibility (or resistance) to ESCs. We also identified ESC resistance determinants and their association with susceptibility of *N. gonorrhoeae* strains to ertapenem. Multiantigen sequence typing (NG-MAST) of *N. gonorrhoeae* isolates was performed to assess distribution according to ertapenem MICs and, potentially, to identify clonality of isolates with increased resistance.

## RESULTS

### Antimicrobial susceptibility.

A total of 259 *N. gonorrhoeae* isolates with decreased susceptibility or resistance to ceftriaxone and/or cefixime were identified. The MIC ranges of ceftriaxone and cefixime for these isolates were 0.06-1 mg/L ($MIC_{50}$, 0.125 mg/L and $MIC_{90}$, 0.125 mg/L) and 0.06-≥4 mg/L ($MIC_{50}$, 0.125 mg/L and $MIC_{90}$, 0.5 mg/L), respectively. Among these isolates, 9 (3.5%) were fully resistant to ceftriaxone (MICs ≥0.5 mg/L) and cefixime (MICs ≥2 mg/L).

MICs of ertapenem against the 259 isolates ranged from 0.006 mg/L to 0.38 mg/L; $MIC_{50}$ and $MIC_{90}$ were 0.032 mg/L and 0.125 mg/L. For the 9 *N. gonorrhoeae* isolates fully resistant to ceftriaxone (MICs ≥ 0.5 mg/L) and cefixime (MICs ≥ 2 mg/L), the ertapenem $MIC_{50}$, $MIC_{90}$ and MIC range were 0.094, 0.19 and 0.023-0.19 mg/L, respectively. 44 (17.0%) isolates had ertapenem MICs ≥ 0.125 mg/L); 10 (3.9%) had MICs ≥ 0.25 mg/L, MICs that represent the

WHO-recommended susceptibility breakpoints for ceftriaxone and cefixime, respectively. The ertapenem $MIC_{50}$ and $MIC_{90}$ increased from 0.023 mg/L and 0.047 mg/L in 2013 to 0.047 mg/L and 0.125 mg/L in 2019, respectively. The distributions of ertapenem MICs during 2013–2019 are shown in Figure 1. The proportion of isolates with ertapenem MICs ≥ 0.125 mg/L (the breakpoint against ceftriaxone) increased from 4.0% in 2013 to 20.0% in 2019, showing an overall upward trend during the study period ($\chi^2$= 24.144, P<0.001; Chi square test for linear trend), while the percent of isolates with MICs ≤ 0.012 mg/L declined in each successive year, sequentially ($\chi^2$= 23.634, P<0.001; Chi square test for linear trend).

**Genetic resistance determinants (*penA*, *mtrR*, *penB* and *ponA*) of ESCs**

A *penA* mosaic allele was present in 118 (45.6%) *N. gonorrhoeae* isolates with decreased susceptibility or resistance to ESCs; non-mosaic *penA* alleles with A501V/T mutations were present in 139 (53.7%); the remaining 2 isolates (0.8%) possessed a non-mosaic allele with an A517G mutation. Mutations in the promoter and/or coding regions of the *mtrR* gene were identified in 179 (69.1%) isolates. Amino acid substitutions at residue G120 of the *penB* gene were present in 5 (1.9%) isolates; G120/A121 double mutations were present in 253 (97.7%). An L421P mutation in the *ponA* gene was present in 256 (98.8%) isolates.

Ertapenem susceptibilities of isolates containing the *penA* mosaic allele, were lower compared to susceptibilities of isolates that lacked the mosaic allele. The $MIC_{50}$,

133 $MIC_{90}$ and the MIC range of ertapenem in strains with the *penA* mosaic allele were
134 0.047 mg/L, 0.19 mg/L and 0.008-0.38 mg/L, respectively. Strains that lacked the
135 mosaic allele had $MIC_{50}$, $MIC_{90}$ and an MIC range of ertapenem of 0.016 mg/L, 0.064
136 mg/L and 0.006–0.125 mg/L, respectively. The *penA* mosaic allele was more common
137 among isolates with increased ertapenem MICs (≥ 0.125 mg/L) (WHO-recommended
138 susceptibility breakpoint against ceftriaxone) vs. isolates with MICs ≤ 0.094 mg/L
139 (97.7% vs. 34.9%, respectively; $\chi^2$=58.158, P<0.001; Table 1). All isolates with
140 ertapenem MICs ≥ 0.25 mg/L (WHO-recommended susceptibility breakpoint against
141 cefixime) possessed the *penA* mosaic allele. Conversely, the proportion of isolates
142 with ertapenem MICs ≤ 0.094 mg/L that possessed A501V/T mutations, specifically,
143 was higher than in isolates with MICs ≥ 0.125 mg/L (64.2% vs. 2.3%, respectively;
144 $\chi^2$=56.307, P<0.001; Table 1). The two isolates with A517G mutations had ertapenem
145 MICs of ≤ 0.094 mg/L (Table 1).
146
147 *mtrR* mutations were present in 34.1% (15/44) of isolates with ertapenem MICs ≥
148 0.125 mg/L and in 76.3% (164/215) of isolates with ertapenem MICs ≤0.094 mg/L
149 ($\chi^2$= 30.453, P<0.001). A single A-deletion in the *mtrR* promoter was identified more
150 often in isolates with ertapenem MICs ≤ 0.094 mg/L than in isolates with MICs ≥
151 0.125 mg/L ($\chi^2$= 9.090, P=0.0026; Table 1). There were no significant differences in
152 the rates of A39T or G45D *mtrR* mutations in the coding region accompanied (or not)
153 by an A deletion in the promoter region. An exception was a G45D mutation
154 accompanied by an A deletion in the promoter, which accounted for 11.2% (24/215)

of isolates with ertapenem MICs ≤ 0.094 mg/L and no isolates with ertapenem MICs ≥ 0.125 mg/L ($\chi^2$= 5.413, P=0.0186; Table 1). All but two isolates with ertapenem MICs ≥ 0.25mg/L lacked the *mtrR* mutations; the two exceptions harbored a single A-deletion in the *mtrR* promoter or G45D mutation in the *mtrR* coding region.

### *N. gonorrhoeae* multiantigen sequence typing (NG-MAST)

The 259 *N. gonorrhoeae* isolates were assigned to 161 NG-MAST types, of which 68 have not been reported previously in the NG-MAST database (www.ng-mast.net). The most prevalent NG-MAST sequence type (ST) was ST5308 (n = 22; ertapenem $MIC_{50}$, 0.094mg/L), followed by ST7554 (n=17; ertapenem $MIC_{50}$, 0.032 mg/L), ST3356 (n = 7; ertapenem $MIC_{50}$, 0.023 mg/L), ST270 (n = 7; ertapenem $MIC_{50}$, 0.008 mg/L), and ST4539 (n = 7; ertapenem $MIC_{50}$, 0.016 mg/L). Among all sequence types, ST5308 was predominant among isolates with MICs ≥ 0.125 mg/L to ertapenem (10/44 [22.7%]); these isolates also showed the highest ertapenem $MIC_{50}$ (0.094mg/L). Furthermore, ST5308 was more common among isolates with MICs ≥ 0.125 mg/L to ertapenem vs. isolates with MICs ≤ 0.094 mg/L (22.7% and 5.6% respectively; $\chi^2$=13.815, P=0.001). All ST5308 isolates had a *penA* mosaic allele, G120K plus A121D substitutions in *penB* and L421P in *ponA* but no *mtrR* mutations.

## DISCUSSION

*Neisseria gonorrhoeae* is becoming increasingly resistant to currently used antimicrobial agents with the real prospect that untreatable gonorrhea may soon

177 appear (9, 13). In the context of limited treatment options, alternative antimicrobials,
178 new and repurposed, are needed urgently to ensure future successful treatments.
179 Ertapenem is a member of the carbapenem family of antibiotics with a broad
180 spectrum of activity. It is active against a variety of gram-positive and -negative
181 bacteria, including *N. gonorrhoeae*. Ertapenem has been used successfully to treat *N.*
182 *gonorrhoeae* that possessed combined high-level azithromycin and ceftriaxone
183 resistance (13). In our study, the $MIC_{50}$ of ertapenem (0.032 mg/L) was substantially
184 lower than those observed for both ceftriaxone and cefixime (0.125 mg/L). The $MIC_{90}$
185 of ertapenem (0.125 mg/L) was similar to the $MIC_{90}$ observed for ceftriaxone (0.125
186 mg/L), but lower than the cefixime $MIC_{90}$ (0.5 mg/L). Unemo et al (17) reported in
187 2012, that generally, ertapenem and ceftriaxone $MIC_{50}$s and $MIC_{90}$s were similar
188 (0.032mg/L [both] and 0.64 mg/L [ertapenem] /0.125 mg/L [ceftriaxone], respectively)
189 in 257 *N. gonorrhoeae* clinical isolates with highly diverse ceftriaxone MIC values
190 referred to WHO Collaborating Centres for STIs. Similarly, ertapenem MIC ranges
191 were lower than MIC ranges for ceftriaxone and cefixime in our study, also reported
192 by Unemo et al (17). In our study, 83.0%/ 96.1% of isolates had ertapenem MICs
193 below the ceftriaxone/cefixime breakpoints (0.125mg/L / 0.25 mg/L), similar to the
194 study by Xu et al (22) that examined gonococcal isolates from eight provinces in
195 China. In that study, 83.3% of 24 isolates with decreased susceptibility to ceftriaxone
196 (MIC $\geq$ 0.25 mg/L) exhibited ertapenem MIC values <0.25 mg/L, the cefixime
197 breakpoint. Unemo et al (17) reported that all strains had ertapenem MICs $\leq$ 0.125
198 mg/L, the ceftriaxone breakpoint. These results predict that ertapenem may be

199 uniformly effective clinically in most instances because higher MICs are infrequent
200 (our study and the study by Xu et al (22)) or absent altogether (17). Further support
201 for clinical efficacy is derived from activity of ertapenem against two extensively
202 drug resistant (XDR) *N. gonorrhoeae* strains, H041 and F89; both are highly resistant
203 to cefixime (MIC range, 4-8 mg/L) and ceftriaxone (MIC range, 2-4 mg/L) (17).
204 Ertapenem MICs were reported to be significantly lower (0.064 mg/L and 0.016 mg/L)
205 for these two strains, respectively (17), corroborated in a separate study where F89
206 had an ertapenem MIC of 0.03 mg/L (23). In our study, ertapenem was also effective
207 against the 9 *N. gonorrhoeae* isolates fully resistant to ceftriaxone (MICs $\geq$ 0.5 mg/L)
208 and cefixime (MICs $\geq$ 2 mg/L). Nonetheless, these studies (17) (23) and another (24)
209 have shown that ertapenem had no apparent *in vitro* advantage over ceftriaxone for *N.*
210 *gonorrhoeae* isolates with lower ceftriaxone MICs.
211
212 Similar to other β-lactam antimicrobials, reduced activity of ertapenem against some
213 bacteria is mediated by: mutations in porin that result in aberrant function (25);
214 upregulation of efflux pumps (26) and production of carbapenemases (27). However,
215 resistance of *N. gonorrhoeae* to ertapenem is not fully defined. For example, we
216 found that, *penB* and *ponA* resistance determinants were present across most strains,
217 perhaps without a meaningful effect on ertapenem susceptibility. We also found that
218 the presence of a *penA* mosaic allele was strongly associated with increased MICs of
219 ertapenem, similar to findings reported by Unemo et al (17). However, our result
220 showed that *mtrR* mutations were present in a higher percentage of isolates with

ertapenem MICs ≤ 0.094mg/L than in isolates with ertapenem MICs ≥ 0.125mg/L, different from another Chinese study, which showed that the *mtrR* promoter A-deletion was significantly associated with strains displaying an ertapenem MIC > 0.125 mg/L (28).

NG-MAST has been evaluated as a tool for predicting specific antimicrobial resistance phenotypes in *N. gonorrhoeae* isolates (29, 30). In our study, ST5308 was the most prevalent NG-MAST sequence type (ST) among the 259 isolates with decreased susceptibility or resistance to ESCs. In addition, ST5308 was the most highly represented ST in isolates with increased ertapenem MICs (≥ 0.125mg/L). ST5308 isolates, possessing a *penA* mosaic allele, have been reported in Hong Kong, and were associated with decreased susceptibility to oral ESCs (31). Between 2013 and 2017, ST5308 was the most common gonococcal type isolated in Guangdong, China (32).

In summary, *in vitro* susceptibility to ertapenem of *Neisseria gonorrhoeae* isolates with decreased susceptibility (or resistance) to ESCs, suggests potential for future use of ertapenem as treatment for antimicrobial resistant infections. However, the *penA* mosaic allele, commonly associated with ESC resistance, was also associated with increased MICs of ertapenem. Continued surveillance of antimicrobial susceptibility of ertapenem supplemented by sequence typing and NG-MAST classification are warranted.

## MATERIALS AND METHODS

### Bacterial strains

From January 2013 to December 2019, a total of 1321 *N. gonorrhoeae* strains were isolated from men with symptomatic urethritis (urethral discharge and/or dysuria) attending the STD clinic at the Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, Jiangsu Province, China. Urethral exudates were collected with cotton swabs and immediately streaked on to modified Thayer-Martin (T-M) selective medium (Zhuhai DL Biotech Co. Ltd.) and incubated at 36°C in candle jars for 24–48 h. *N. gonorrhoeae* was identified by colonial morphology, Gram's stain, and oxidase testing, which are sufficient to identify *N. gonorrhoeae* colonies isolated on selective medium, particularly for urethral samples from symptomatic men (33, 34). Gonococcal isolates were subcultured onto chocolate agar plates; pure colonies were swabbed, suspended in tryptone-based soy broth and frozen (−80°C) until used for antimicrobial testing.

### Antimicrobial susceptibility testing

Susceptibility testing for ceftriaxone and cefixime was performed by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (35). According to criteria for decreased susceptibility or resistance to ceftriaxone (MIC $\geq$ 0.125 mg/L) and cefixime (MIC $\geq$ 0.25 mg/L), defined by WHO (36), 259 strains were eligible for inclusion in this study. Ertapenem susceptibility among these

265 isolates was determined by Etest (Liofilchem®, Italy) method, according to the
266 manufacturer's instructions (37). Strain WHO-P was used for quality control. No
267 interpretative criteria have been provided by CLSI (or any other organization) for
268 ertapenem susceptibility breakpoints against *N. gonorrhoeae*.
269
270 **Sequencing of resistance determinants (*penA*, *mtrR*, *penB* and *ponA*) and *N.***
271 ***gonorrhoeae* multiantigen sequence typing (NG-MAST)**
272 Genomic DNA was prepared from individual gonococcal isolates using the Rapid
273 Bacterial Genomic DNA Isolation Kit (DNA-EZ Reagents V All-DNA-Fast-Out,
274 Sangon Biotech Co. Ltd, Shanghai). ESCs resistance determinants: *penA*; *mtrR*; *penB*
275 and *ponA* were amplified by PCR using published primers (38) and DNA sequencing
276 performed by Suzhou Genewiz Biotech Co. Ltd. The sequencing data was uploaded to
277 the NG-STAR database (https://ngstar.canada.ca) to determine the ESCs resistance
278 determinants.
279
280 Genetic characterization was performed by *N. gonorrhoeae* multiantigen sequence
281 typing (NG-MAST), which assigns sequence types (STs) based on a combination of
282 two variable genes: *porB* and *tbpB* (39); allele numbers and sequence types (STs)
283 were then assigned (http://www.ng-mast.net).
284
285 **Data Analysis**
286 Chi-square ($\chi^2$) testing for linear trends was used to assess changes in ertapenem

MICs during the study period. Chi-square or Fisher exact testing was used to determine the associations between ertapenem susceptibility and gonococcal genetic resistance determinants or *N. gonorrhoeae* multi-antigen sequence types. SPSS version 26.0 was used for statistical analysis and P values <0.05 considered significant.

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## CONFLICTS OF INTEREST

No conflicts for all authors.

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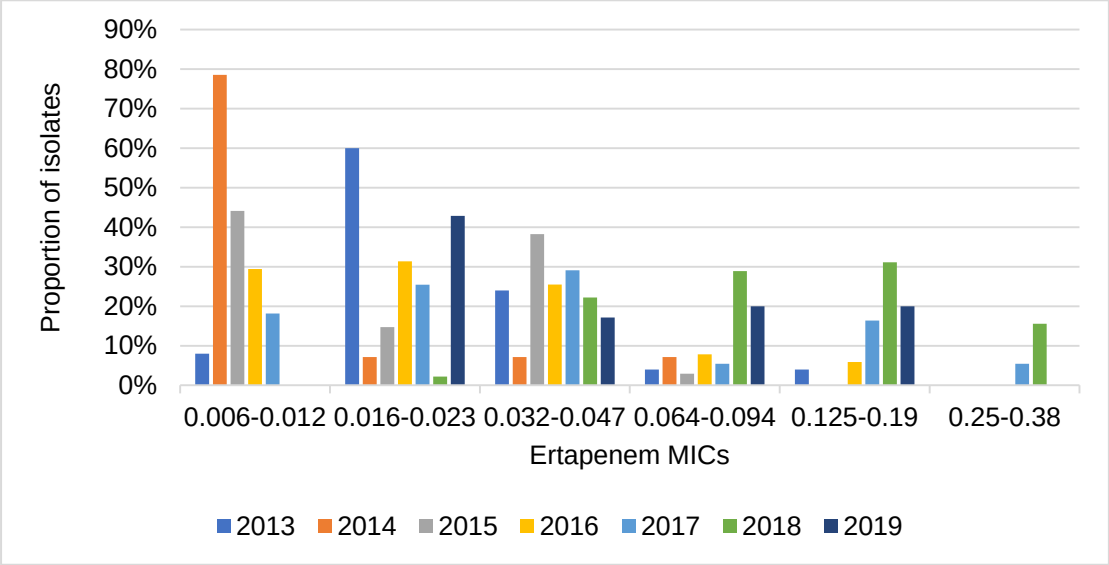


Figure 1. Distribution of ertapenem MICs for 259 *Neisseria gonorrhoeae* clinical isolates with decreased susceptibility (or resistance) to ESCs isolated in Nanjing, China, 2013–2019.

457 Table 1. *penA* and *mtrR* mutations in isolates either with MICs ≤0.094mg/L to

458 ertapenem or MICs to ertapenem ≥0.125mg/L.

| Resistance determinants | Number / proportion of isolates | | $\chi^2$ | P value [a] |
|---|---|---|---|---|
| | MIC≤0.094mg/L (N=215) | MIC≥0.125mg/L (N=44) | | |
| ***penA*** | | | | |
| Mosaic allele | 75 (34.9%) | 43 (97.7%) | 58.158 | <0.001 |
| A501V/T [b] | 138 (64.2%) | 1 (2.3%) | 56.307 | <0.001 |
| A517G [b] | 2(0.9%) | 0 | 0.413 | 1 |
| ***mtrR*** | | | | |
| A-deletion in promoter region [c] | 102 (47.4%) | 10 (22.7%) | 9.090 | 0.0026 |
| A-deletion [c], A39T | 3 (1.4%) | 3 (6.8%) | 4.747 | 0.0632 |
| A-deletion [c], G45D | 24 (11.2%) | 0 (0) | 5.413 | 0.0186 |
| A39T | 8 (3.7%) | 1 (2.3%) | 0.228 | 1.0000 |
| G45D | 27 (12.6%) | 1 (2.3%) | 4.007 | 0.0585 |
| WT | 51 (23.7%) | 29 (65.9%) | 30.453 | <0.001 |

459 [a] P<0.05 was considered significant in Chi-square ($\chi^2$) or Fisher exact testing.

460 [b] Non-mosaic *penA* alleles.

461 [c]A (adenine) deletion in the 13-bp inverted-repeat sequence of the *mtrR* promoter.