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

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15 **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) $\geq$ 0.125 mg/L and $\geq$ 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 epilometer 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 MIC₅₀ and MIC₉₀ were 0.032 mg/L and 0.125 mg/L. 44 (17.0%) isolates displayed ertapenem MICs of $\geq$ 0.125 mg/L; 10 (3.9%) had MICs $\geq$ 0.25 mg/L. The proportion of isolates with ertapenem MICs $\geq$ 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 $\geq$ 0.125 mg/L compared to isolates with MICs $\leq$ 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 ≤ 0.094 mg/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

therapies have been reported (12, 13). Thus, currently recommended treatments are unlikely to continue to be effective long-term; exploring novel or repurposed antimicrobials will be essential for control of gonorrhea (7).

Ertapenem is a parenteral carbapenem, effective against gram-negative bacteria that may, otherwise, be resistant to cephalosporins. Similar to other β -lactams, ertapenem inhibits cell wall synthesis by binding to and inhibiting penicillin-binding proteins (PBPs) (14). It is well tolerated, effective and has a safety profile comparable to that of ceftriaxone (15, 16). Ertapenem has been used successfully to treat *N. gonorrhoeae* with both high-level azithromycin and ceftriaxone resistance (13) and may be an effective treatment option for gonorrhea, particularly infections caused by strains resistant to extended spectrum cephalosporins (ESCs).

No specific genetic determinants of ertapenem resistance or carbapenemases, generally, have been identified in *N. gonorrhoeae*; however, there may be overlap with resistant mechanisms exhibited by other ESCs (17). Mechanisms of resistance against ESCs can result from amino acid changes caused by nucleotide mutations in: *penA* (encoding penicillin-binding protein 2, PBP2); *mtrR* (encoding the multiple transfer resistance repressor, MtrR); *penB* (encoding porin PorB) and *ponA* (encoding penicillin-binding protein 1, PBP1) in *N. gonorrhoeae* (3, 18-21).

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 (MIC₅₀, 0.125 mg/L and MIC₉₀, 0.125 mg/L) and 0.06-≥4 mg/L (MIC₅₀, 0.125 mg/L and MIC₉₀, 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; MIC₅₀ and MIC₉₀ 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 MIC₅₀, MIC₉₀ 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 MIC₅₀ and MIC₉₀ 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 $\geq$ 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 $\leq$ 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 MIC₅₀,

MIC₉₀ and the MIC range of ertapenem in strains with the *penA* mosaic allele were 0.047 mg/L, 0.19 mg/L and 0.008-0.38 mg/L, respectively. Strains that lacked the mosaic allele had MIC₅₀, MIC₉₀ and an MIC range of ertapenem of 0.016 mg/L, 0.064 mg/L and 0.006–0.125 mg/L, respectively. The *penA* mosaic allele was more common among isolates with increased ertapenem MICs (≥ 0.125 mg/L) (WHO-recommended susceptibility breakpoint against ceftriaxone) vs. isolates with MICs ≤ 0.094 mg/L (97.7% vs. 34.9%, respectively; $\chi^2=58.158$, $P<0.001$; Table 1). All isolates with ertapenem MICs ≥ 0.25 mg/L (WHO-recommended susceptibility breakpoint against cefixime) possessed the *penA* mosaic allele. Conversely, the proportion of isolates with ertapenem MICs ≤ 0.094 mg/L that possessed A501V/T mutations, specifically, was higher than in isolates with MICs ≥ 0.125 mg/L (64.2% vs. 2.3%, respectively; $\chi^2=56.307$, $P<0.001$; Table 1). The two isolates with A517G mutations had ertapenem MICs of ≤ 0.094 mg/L (Table 1).

mtrR mutations were present in 34.1% (15/44) of isolates with ertapenem MICs ≥ 0.125 mg/L and in 76.3% (164/215) of isolates with ertapenem MICs ≤ 0.094 mg/L ($\chi^2= 30.453$, $P<0.001$). A single A-deletion in the *mtrR* promoter was identified more often in isolates with ertapenem MICs ≤ 0.094 mg/L than in isolates with MICs ≥ 0.125 mg/L ($\chi^2= 9.090$, $P=0.0026$; Table 1). There were no significant differences in the rates of A39T or G45D *mtrR* mutations in the coding region accompanied (or not) by an A deletion in the promoter region. An exception was a G45D mutation 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 ≥ 0.25 mg/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 MIC₅₀, 0.094 mg/L), followed by ST7554 ($n = 17$; ertapenem MIC₅₀, 0.032 mg/L), ST3356 ($n = 7$; ertapenem MIC₅₀, 0.023 mg/L), ST270 ($n = 7$; ertapenem MIC₅₀, 0.008 mg/L), and ST4539 ($n = 7$; ertapenem MIC₅₀, 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 MIC₅₀ (0.094 mg/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

appear (9, 13). In the context of limited treatment options, alternative antimicrobials, new and repurposed, are needed urgently to ensure future successful treatments. Ertapenem is a member of the carbapenem family of antibiotics with a broad spectrum of activity. It is active against a variety of gram-positive and -negative bacteria, including *N. gonorrhoeae*. Ertapenem has been used successfully to treat *N. gonorrhoeae* that possessed combined high-level azithromycin and ceftriaxone resistance (13). In our study, the MIC₅₀ of ertapenem (0.032 mg/L) was substantially lower than those observed for both ceftriaxone and cefixime (0.125 mg/L). The MIC₉₀ of ertapenem (0.125 mg/L) was similar to the MIC₉₀ observed for ceftriaxone (0.125 mg/L), but lower than the cefixime MIC₉₀ (0.5 mg/L). Unemo et al (17) reported in 2012, that generally, ertapenem and ceftriaxone MIC₅₀s and MIC₉₀s were similar (0.032mg/L [both] and 0.64 mg/L [ertapenem] /0.125 mg/L [ceftriaxone], respectively) in 257 *N. gonorrhoeae* clinical isolates with highly diverse ceftriaxone MIC values referred to WHO Collaborating Centres for STIs. Similarly, ertapenem MIC ranges were lower than MIC ranges for ceftriaxone and cefixime in our study, also reported by Unemo et al (17). In our study, 83.0%/ 96.1% of isolates had ertapenem MICs below the ceftriaxone/cefixime breakpoints (0.125mg/L / 0.25 mg/L), similar to the study by Xu et al (22) that examined gonococcal isolates from eight provinces in China. In that study, 83.3% of 24 isolates with decreased susceptibility to ceftriaxone (MIC $\geq$ 0.25 mg/L) exhibited ertapenem MIC values <0.25 mg/L, the cefixime breakpoint. Unemo et al (17) reported that all strains had ertapenem MICs $\leq$ 0.125 mg/L, the ceftriaxone breakpoint. These results predict that ertapenem may be

uniformly effective clinically in most instances because higher MICs are infrequent (our study and the study by Xu et al (22)) or absent altogether (17). Further support for clinical efficacy is derived from activity of ertapenem against two extensively drug resistant (XDR) *N. gonorrhoeae* strains, H041 and F89; both are highly resistant to cefixime (MIC range, 4-8 mg/L) and ceftriaxone (MIC range, 2-4 mg/L) (17). Ertapenem MICs were reported to be significantly lower (0.064 mg/L and 0.016 mg/L) for these two strains, respectively (17), corroborated in a separate study where F89 had an ertapenem MIC of 0.03 mg/L (23). In our study, ertapenem was also effective against the 9 *N. gonorrhoeae* isolates fully resistant to ceftriaxone (MICs $\geq$ 0.5 mg/L) and cefixime (MICs $\geq$ 2 mg/L). Nonetheless, these studies (17) (23) and another (24) have shown that ertapenem had no apparent *in vitro* advantage over ceftriaxone for *N. gonorrhoeae* isolates with lower ceftriaxone MICs.

Similar to other β -lactam antimicrobials, reduced activity of ertapenem against some bacteria is mediated by: mutations in porin that result in aberrant function (25); upregulation of efflux pumps (26) and production of carbapenemases (27). However, resistance of *N. gonorrhoeae* to ertapenem is not fully defined. For example, we found that, *penB* and *ponA* resistance determinants were present across most strains, perhaps without a meaningful effect on ertapenem susceptibility. We also found that the presence of a *penA* mosaic allele was strongly associated with increased MICs of ertapenem, similar to findings reported by Unemo et al (17). However, our result showed that *mtrR* mutations were present in a higher percentage of isolates with

ertapenem MICs $\leq 0.094\text{mg/L}$ than in isolates with ertapenem MICs $\geq 0.125\text{mg/L}$,
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 ($\geq 0.125\text{mg/L}$).
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.

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244 MATERIALS AND METHODS

245 Bacterial strains

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

258

259 Antimicrobial susceptibility testing

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

isolates was determined by Etest (Liofilchem®, Italy) method, according to the manufacturer's instructions (37). Strain WHO-P was used for quality control. No interpretative criteria have been provided by CLSI (or any other organization) for ertapenem susceptibility breakpoints against *N. gonorrhoeae*.

Sequencing of resistance determinants (*penA*, *mtrR*, *penB* and *ponA*) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST)

Genomic DNA was prepared from individual gonococcal isolates using the Rapid Bacterial Genomic DNA Isolation Kit (DNA-EZ Reagents V All-DNA-Fast-Out, Sangon Biotech Co. Ltd, Shanghai). ESCs resistance determinants: *penA*; *mtrR*; *penB* and *ponA* were amplified by PCR using published primers (38) and DNA sequencing performed by Suzhou Genewiz Biotech Co. Ltd. The sequencing data was uploaded to the NG-STAR database (<https://ngstar.canada.ca>) to determine the ESCs resistance determinants.

Genetic characterization was performed by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), which assigns sequence types (STs) based on a combination of two variable genes: *porB* and *tbpB* (39); allele numbers and sequence types (STs) were then assigned (<http://www.ng-mast.net>).

Data Analysis

Chi-square (χ^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.

REFERENCES

1. World Health Organization. 2018. Report on global sexually transmitted infection surveillance -2018. World Health Organization, Geneva, Switzerland.
2. Yue, X., X. Gong, J. Li, Y. Wang, and H. Gu. 2019. Gonorrhea in China, 2018. International Journal of Dermatology and Venereology 2:65-69.
3. Ohnishi, M., D. Golparian, K. Shimuta, T. Saika, S. Hoshina, K. Iwasaku, S. Nakayama, J. Kitawaki, and M. Unemo. 2011. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob Agents Chemother 55:3538-45.

- 310 4. Unemo, M., D. Golparian, R. Nicholas, M. Ohnishi, A. Gallay, and P. Sednaoui.
311 2012. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in
312 France: novel penA mosaic allele in a successful international clone causes treatment
313 failure. Antimicrob Agents Chemother 56:1273-80.
- 314 5. Camara, J., J. Serra, J. Ayats, T. Bastida, D. Carnicer-Pont, A. Andreu, and C.
315 Ardanuy. 2012. Molecular characterization of two high-level ceftriaxone-resistant
316 *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. J Antimicrob Chemother
317 67:1858-60.
- 318 6. Unemo, M., C. S. Bradshaw, J. S. Hocking, H. de Vries, S. C. Francis, D. Mabey, J.
319 M. Marrazzo, G. Sonder, J. R. Schwebke, E. Hoornenborg, R. W. Peeling, S. S.
320 Philip, N. Low, and C. K. Fairley. 2017. Sexually transmitted infections: challenges
321 ahead. Lancet Infect Dis 17:e235-e279.
- 322 7. Wi, T., M. M. Lahra, F. Ndowa, M. Bala, J. R. Dillon, P. Ramon-Pardo, S. R.
323 Eremin, G. Bolan, and M. Unemo. 2017. Antimicrobial resistance in *Neisseria*
324 *gonorrhoeae*: Global surveillance and a call for international collaborative action.
325 PLoS Med 14:e1002344.
- 326 8. World Health Organization. 2016.WHO guidelines for the treatment of *Neisseria*
327 *gonorrhoeae*. World Health Organization, Geneva, Switzerland.
- 328 9. Unemo, M., and W. M. Shafer. 2014. Antimicrobial resistance in *Neisseria*
329 *gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev
330 27:587-613.
- 331 10. St, C. S., L. Barbee, K. A. Workowski, L. H. Bachmann, C. Pham, K. Schlanger,

332 E. Torrone, H. Weinstock, E. N. Kersh, and P. Thorpe. 2020. Update to CDC's
333 Treatment Guidelines for Gonococcal Infection, 2020. MMWR Morb Mortal Wkly
334 Rep 69:1911-1916.

335 11. Yan, J., J. Xue, Y. Chen, S. Chen, Q. Wang, C. Zhang, S. Wu, H. Lv, Y. Yu, and
336 S. van der Veen. 2019. Increasing prevalence of *Neisseria gonorrhoeae* with
337 decreased susceptibility to ceftriaxone and resistance to azithromycin in Hangzhou,
338 China (2015-17). J Antimicrob Chemother 74:29-37.

339 12. Fifer, H., U. Natarajan, L. Jones, S. Alexander, G. Hughes, D. Golparian, and M.
340 Unemo. 2016. Failure of Dual Antimicrobial Therapy in Treatment of Gonorrhea. N
341 Engl J Med 374:2504-6.

342 13. Eyre, D. W., N. D. Sanderson, E. Lord, N. Regisford-Reimmer, K. Chau, L.
343 Barker, M. Morgan, R. Newnham, D. Golparian, M. Unemo, D. W. Crook, T. E. Peto,
344 G. Hughes, M. J. Cole, H. Fifer, A. Edwards, and M. I. Andersson. 2018. Gonorrhoea
345 treatment failure caused by a *Neisseria gonorrhoeae* strain with combined ceftriaxone
346 and high-level azithromycin resistance, England, February 2018. Euro Surveill 23.

347 14. Congeni, B. L. 2010. Ertapenem. Expert Opin Pharmacother 11:669-72.

348 15. Arguedas, A., J. Cespedes, F. A. Botet, J. Blumer, R. Yogev, R. Gesser, J. Wang,
349 J. West, T. Snyder, and W. Wimmer. 2009. Safety and tolerability of ertapenem
350 versus ceftriaxone in a double-blind study performed in children with complicated
351 urinary tract infection, community-acquired pneumonia or skin and soft-tissue
352 infection. Int J Antimicrob Agents 33:163-7.

353 16. Burkhardt, O., H. Derendorf, and T. Welte. 2007. Ertapenem: the new

- 354 carbapenem 5 years after first FDA licensing for clinical practice. *Expert Opin*
355 *Pharmacother* 8:237-56.
- 356 17. Unemo, M., D. Golparian, A. Limnios, D. Whiley, M. Ohnishi, M. M. Lahra, and
357 J. W. Tapsall. 2012. In vitro activity of ertapenem versus ceftriaxone against
358 *Neisseria gonorrhoeae* isolates with highly diverse ceftriaxone MIC values and
359 effects of ceftriaxone resistance determinants: ertapenem for treatment of gonorrhea?
360 *Antimicrob Agents Chemother* 56:3603-9.
- 361 18. Golparian, D., B. Hellmark, H. Fredlund, and M. Unemo. 2010. Emergence,
362 spread and characteristics of *Neisseria gonorrhoeae* isolates with in vitro decreased
363 susceptibility and resistance to extended-spectrum cephalosporins in Sweden. *Sex*
364 *Transm Infect* 86:454-60.
- 365 19. Ison, C. A., J. Hussey, K. N. Sankar, J. Evans, and S. Alexander. 2011.
366 Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. *Euro*
367 *Surveill* 16.
- 368 20. Lindberg, R., H. Fredlund, R. Nicholas, and M. Unemo. 2007. *Neisseria*
369 *gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone:
370 association with genetic polymorphisms in penA, mtrR, porB1b, and ponA.
371 *Antimicrob Agents Chemother* 51:2117-22.
- 372 21. Zhao, S., M. Duncan, J. Tomberg, C. Davies, M. Unemo, and R. A. Nicholas.
373 2009. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and
374 cefixime in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 53:3744-51.
- 375 22. Xu, W. Q., X. L. Zheng, J. W. Liu, Q. Zhou, X. Y. Zhu, J. Zhang, Y. Han, K.

376 Chen, S. C. Chen, X. S. Chen, and Y. P. Yin. 2021. Antimicrobial Susceptibility of
 377 Ertapenem in *Neisseria gonorrhoeae* Isolates Collected Within the China Gonococcal
 378 Resistance Surveillance Programme (China-GRSP) 2018. *Infect Drug Resist*
 379 14:4183-4189.

380 23. Quaye, N., M. J. Cole, and C. A. Ison. 2014. Evaluation of the activity of
 381 ertapenem against gonococcal isolates exhibiting a range of susceptibilities to
 382 cefixime. *J Antimicrob Chemother* 69:1568-71.

383 24. Livermore, D. M., S. Alexander, B. Marsden, D. James, M. Warner, E. Rudd, and
 384 K. Fenton. 2004. Activity of ertapenem against *Neisseria gonorrhoeae*. *J Antimicrob*
 385 *Chemother* 54:280-1.

386 25. Jacoby, G. A., D. M. Mills, and N. Chow. 2004. Role of beta-lactamases and
 387 porins in resistance to ertapenem and other beta-lactams in *Klebsiella pneumoniae*.
 388 *Antimicrob Agents Chemother* 48:3203-6.

389 26. Szabo, D., F. Silveira, A. M. Hujer, R. A. Bonomo, K. M. Hujer, J. W. Marsh, C.
 390 R. Bethel, Y. Doi, K. Deeley, and D. L. Paterson. 2006. Outer membrane protein
 391 changes and efflux pump expression together may confer resistance to ertapenem in
 392 *Enterobacter cloacae*. *Antimicrob Agents Chemother* 50:2833-5.

393 27. McGettigan, S. E., K. Andreacchio, and P. H. Edelstein. 2009. Specificity of
 394 ertapenem susceptibility screening for detection of *Klebsiella pneumoniae*
 395 carbapenemases. *J Clin Microbiol* 47:785-6.

396 28. Yang, F., J. Yan, J. Zhang, and S. van der Veen. 2020. Evaluation of alternative
 397 antibiotics for susceptibility of gonococcal isolates from China. *Int J Antimicrob*

398 Agents 55:105846.

399 29. Palmer, H. M., H. Young, C. Graham, and J. Dave. 2008. Prediction of antibiotic
400 resistance using *Neisseria gonorrhoeae* multi-antigen sequence typing. Sex Transm
401 Infect 84:280-4.

402 30. Thakur, S. D., P. N. Levett, G. B. Horsman, and J. A. Dillon. 2014. Molecular
403 epidemiology of *Neisseria gonorrhoeae* isolates from Saskatchewan, Canada: utility
404 of NG-MAST in predicting antimicrobial susceptibility regionally. Sex Transm Infect
405 90:297-302.

406 31. Lo, J. Y., K. M. Ho, and A. C. Lo. 2012. Surveillance of gonococcal
407 antimicrobial susceptibility resulting in early detection of emerging resistance. J
408 Antimicrob Chemother 67:1422-6.

409 32. Qin, X., Y. Zhao, W. Chen, X. Wu, S. Tang, G. Li, Y. Yuqi, W. Cao, X. Liu, J.
410 Huang, J. Yang, W. Chen, W. Tang, and H. Zheng. 2019. Changing antimicrobial
411 susceptibility and molecular characterisation of *Neisseria gonorrhoeae* isolates in
412 Guangdong, China: in a background of rapidly rising epidemic. Int J Antimicrob
413 Agents 54:757-765.

414 33. Ison, C. A. 1990. Laboratory methods in genitourinary medicine. Methods of
415 diagnosing gonorrhoea. Genitourin Med 66:453-9.

416 34. Unemo M and Ison C. 2013. Gonorrhoea, p21-54. In Laboratory diagnosis of
417 sexually transmitted infections, including human immunodeficiency virus. World
418 Health Organization (WHO), Geneva, Switzerland.

419 35. Clinical and Laboratory Standards Institute. 2012. Performance standards for

antimicrobial susceptibility testing; 22nd informational supplement. CLSI document
M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA.

36. World Health Organization (WHO), Department of Reproductive Health and
Research. 2012. Global action plan to control the spread and impact of antimicrobial
resistance in *Neisseria gonorrhoeae*, p 1–36. WHO, Geneva, Switzerland.

37. AB Biodisk. Etest application sheet. M0000503-MH0184 AB Biodisk, Solna,
Sweden 2007. Available from:
<http://www.illexmedical.com/files/EtestApplicationSheets.pdf>.

38. Demczuk, W., S. Sidhu, M. Unemo, D. M. Whiley, V. G. Allen, J. R. Dillon, M.
Cole, C. Seah, E. Trembizki, D. L. Trees, E. N. Kersh, A. J. Abrams, H. de Vries, A.
P. van Dam, I. Medina, A. Bharat, M. R. Mulvey, G. Van Domselaar, and I. Martin.
2017. *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance, a Novel
Antimicrobial Resistance Multilocus Typing Scheme for Tracking Global
Dissemination of *N. gonorrhoeae* Strains. J Clin Microbiol 55:1454-1468.

39. Martin, I. M., C. A. Ison, D. M. Aanensen, K. A. Fenton, and B. G. Spratt. 2004.
Rapid sequence-based identification of gonococcal transmission clusters in a large
metropolitan area. J Infect Dis 189:1497-505.

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.

Table 1. *penA* and *mtrR* mutations in isolates either with MICs ≤ 0.094 mg/L to ertapenem or MICs to ertapenem ≥ 0.125 mg/L.

Resistance determinants	Number / proportion of isolates		χ^2	P value ^a
	MIC≤0.094mg/L	MIC≥0.125mg/L		
	(N=215)	(N=44)		
<i>penA</i>				
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
<i>mtrR</i>				
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

^a P<0.05 was considered significant in Chi-square (χ^2) or Fisher exact testing.

^b Non-mosaic *penA* alleles.

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