



Multiplex TaqMan real-time PCR platform for detection of *Neisseria gonorrhoeae* with decreased susceptibility to ceftriaxone

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ABSTRACT

A multiplex TaqMan real-time PCR platform was developed in this study for combined detection of *opa* and/or *porA* genes (identification of *N. gonorrhoeae*) and the key mutations (Ala501Val/Thr/Pro, and/or Gly545Ser) in penicillin-binding protein 2 (PBP2) associated with decreased susceptibility to extended-spectrum cephalosporins (ESCs). Firstly, the specificities of the TaqMan probes/primers for the multiplex TaqMan real time PCR platform were confirmed by Basic Local Alignment Search Tool (BLAST) analysis. Then the multiplex PCR platform was performed on 77 isolates with decreased susceptibility to ceftriaxone (CRO) and 100 isolates with full susceptibility to CRO under universal optimized reaction conditions. As a result, based on cultivation-based matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and antimicrobial susceptibility testing in vitro, the multiplex platform had a sensitivity of 100% and a specificity of 95.0% for identifying cultured isolates of *Neisseria gonorrhoeae* (*N. gonorrhoeae*, NG, GC) with decreased susceptibility to CRO. When directly screening *N. gonorrhoeae* with decreased susceptibility to CRO from clinical urogenital swabs, the multiplex platform offered a sensitivity of 96.1% and a specificity of 95.0%. Therefore, on the basis of sample culture and antimicrobial susceptibility testing in vitro, the multiplex TaqMan real time PCR platform has been proven to be a sensitivity of 100% and a specificity of 95.0% useful tool for screening cultured isolates of *N. gonorrhoeae* with decreased susceptibility to CRO, which can be finished within 2 days.

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1. Introduction

Up till now, the extended-spectrum cephalosporins (ESCs) such as ceftriaxone (CRO) are recommended for empirical antimicrobial monotherapy of gonorrhea in many countries and areas of the world. However, *Neisseria gonorrhoeae* (*N. gonorrhoeae*, NG, GC) isolates with decreased susceptibility to the ESCs have been reported worldwide, especially in China due to the extensive use of the ESCs (Chen et al., 2016; Gianecini et al., 2017; Gong et al., 2016; Nakayama et al., 2016; Thakur et al., 2017).

The molecular mechanisms of decreased susceptibility to the ESCs in *N. gonorrhoeae* are complicated. The substitutions of A311V, G542S, P551S/L, I312M, and V316 T in penicillin-binding protein 2 (PBP2) in *N. gonorrhoeae* were suggested to be associated with decreased susceptibility to CRO in early studies (Takahata et al., 2006; Whaley et al., 2010). Later studies revealed that these mutations were present in both CRO-susceptible (CRO^S) and CRO-decreased-susceptible (CRO^D) isolates, having little effect on decreased CRO susceptibility (Liang et al., 2016; Unemo and Shafer, 2014). Recent studies have demonstrated that the key mutations associated with decreased susceptibility

to ESCs include Ala501Val/Thr/Pro, and/or Gly545Ser (relatively specific for mosaic *penA* alleles) in PBP2 in clinical isolates of *N. gonorrhoeae* (André et al., 2016; Bharat et al., 2015; Chen et al., 2016; Gianecini et al., 2017; Gong et al., 2016; Tomberg et al., 2017; Valentina et al., 2016).

Currently, multiplex TaqMan real time PCR is a rapid and reliable method that can identify microbes and simultaneously detect genetic factors associated with antimicrobial resistance in microbes (Kumari et al., 2018; Peterson et al., 2015; Tabrizi et al., 2017; Valentina et al., 2016). Therefore, a multiplex TaqMan real-time PCR platform was developed in this study for combined detection of *opa* and/or *porA* genes (identification of *N. gonorrhoeae*) and the key mutations (Ala501Val/Thr/Pro, and/or Gly545Ser) in PBP2 associated with decreased susceptibility to ESCs (Vahidnia et al., 2015; Valentina et al., 2016).

2. Materials and methods

2.1. Patients, specimens, and isolates

A total of 897 *N. gonorrhoeae* isolates were collected from patients with gonorrhea in 30 major hospitals in Tai'an, Qingdao, Yantai, Weihai, Linyi, Jinan, Binzhou, Liaocheng, Zibo, Rizhao, Dezhou, and Jinan of Shandong Province in China during 2013–2017. The age range of the

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patients was from 18 to 49 years (mean, 27.7 years), and the ratio of male to female patients was 10:1. The urethral swabs were collected from the male patients with penile discharges and/or dysuria. The cervical swabs were collected from the female patients with vaginal discharges. Urine specimens were not collected and any co-infection with CT was not detected. Sterile cotton-tipped swabs were inserted approximately 2–4 cm into the urethra and rotated for 3–5 seconds for male patients. Sterile cotton-tipped swabs were inserted approximately 1–2 cm into the cervix and rotated for 10–20 seconds for female patients. The swabs were immediately placed in sterile tubes and sent to Clinical Microbiology Laboratory for culture, and then stored at -20°C within 18 hours of collection for direct testing after half a year later. Testing personnel were blinded to results of culture and antibiotic susceptibility testing.

N. gonorrhoeae isolates were cultured on the chocolate agar plates added with PolyViteX and vancomycin (Oxoid Limited, Basingstoke, UK) in a 5% CO_2 incubator at 35°C and relative humidity of 85% for 24 to 48 h. Identification of *N. gonorrhoeae* was achieved by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Autobio Diagnostics Co, Ltd, Zhengzhou, China). *N. gonorrhoeae* isolates were preserved in the sterilized skimmed milk at -70°C .

2.2. Antimicrobial susceptibility testing

Penicillin (PEN), tetracycline (TET), ciprofloxacin (CIP), ceftriaxone (CRO), cefixime (CFM), azithromycin (AZM), and spectinomycin (SPE) were purchased from Oxoid Limited (Basingstoke, Hants, UK). The minimal inhibitory concentration (MIC; mg/L) values of PEN, TET, CIP, CRO, CFM, AZM, and SPE were determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015). The beta-lactamase production was identified using nitrocefin solution (Oxoid, Basingstoke, Hants, England). ATCC 49226 and *N. gonorrhoeae* WHO reference strains G, L, M, O were used as quality controls for MIC determinations (Unemo et al., 2009). All the results were interpreted according to the breakpoints stated by the CLSI except AZM and CRO, which were interpreted according to the breakpoints stated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) criteria (Breakpoint tables for interpretation of MICs and zone diameters, 2017). Notably, the MIC data for CRO that were greater than or equal to the CLSI MIC breakpoint of 0.25 mg/L (MICs ≥ 0.25 mg/L) were also interpreted by the CLSI criteria. CRO MICs of ≥ 0.125 mg/L were defined as decreased susceptibility to CRO by WHO in 2012 (World Health Organization, 2012).

2.3. DNA isolation

Genomic DNA from colonies or clinical specimens was extracted and purified by Wizard Genomic DNA Purification Kit (Promega Wizard, USA) according to the manufacturer's instructions. The extracted DNA was stored at -20°C for later use.

2.4. The control strains

The *N. gonorrhoeae* control strains included WHO reference strains G, L, M, O, strain TA0011, strain TA0787, strain F89, and strain ATCC 49226. WHO reference strain L harbored an Ala501Val substitution in PBP2. TA0011 strain, isolated in Tai'an of China in November 8, 2013, harbored an Ala501Thr substitution (non-mosaic XVIII allele) in PBP2 (Zhao et al., 2018). TA0787 strain, isolated in Jinan of China in September 7, 2014, harbored a Gly545Ser substitution (mosaic *penA* pattern XXXIV allele) in PBP2 (Zhao et al., 2018). F89 strain carried a Gly545Ser substitution (mosaic *penA* pattern XXXIV allele) with an additional Ala501Pro substitution (Valentina et al., 2016). Twenty strains of non-gonococcal *Neisseria* species as negative control strains were cultured using the same procedures for culturing *N. gonorrhoeae* as described

above in the Clinical Microbiology Laboratory in Tai'an Central Hospital. They were identified using the same MALDI-TOF MS (Autobio Diagnostics Co., Ltd. as for identification of *N. gonorrhoeae*. They were preserved in the sterilized skimmed milk at -70°C for later use.

2.5. Development of a multiplex TaqMan real-time PCR platform

The primers and TaqMan probes for the multiplex TaqMan real time PCR platform were designed by using Primer Express, version 3.0 (Applied Biosystems, Foster City, CA, USA), and synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). They were summarized in Table 1. The specificities of the TaqMan probes/primers were confirmed by using Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis, and the multiplex PCR platform was performed on 77 isolates with decreased susceptibility to CRO and 100 isolates with full susceptibility to CRO under universal optimized reaction conditions.

Each 20 μL reaction mixture contained 0.4 μM of each primer, 0.3 μM of TaqMan probe, 1 \times TaqMan genotyping master mix (Life Technologies, USA), and 20 ng of genomic DNA (gDNA). Experiments were run on the ViiA™ 7 Real Time PCR instrument (Life Technologies). Amplification reactions were carried out as follows: denaturation at 95°C for 10 min, and then 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. Results were considered positive if they had a cycle threshold (Ct) value of <35 (Severin et al., 2013) and a relative fluorescence of probe minus the baseline (ΔRn) value of >0.5 (Peterson et al., 2015).

To evaluate the developed multiplex TaqMan real-time PCR, all the screened 77 decreased-CRO-susceptibility isolates harboring Ala501Val/Thr/Pro, and/or Gly545Ser substitutions were detected by the multiplex platform. Meanwhile, 100 randomly-selected full-CRO-susceptibility isolates without Ala501Val/Thr/Pro and Gly545Ser substitutions were used as the control.

2.6. *PenA* gene sequencing and NG-MAST analysis

The primers for amplifying PBP2 were designed and synthesized by Sangon Biotech Co. Ltd. (Table S1). Briefly, 50 μL of reaction mixture contained 1 μL DNA template (100 ng/ μL), 1 μL each primer (10 $\mu\text{mol}/\mu\text{L}$), 1 μL dNTP (10 mM), 5 μL 10 \times PCR Buffer (without Mg^{2+}), 5 μL MgCl_2 (25 mM), 0.5 μL Taq DNA Polymerase (5 U/ μL ; Sangon Biotech.), and 35.5 μL sterile deionized water. PCR was performed as follows: denaturation at 95°C for 3 min; and then 35 cycles of denaturation at 94°C for 30 s, annealing at $55\text{--}60^{\circ}\text{C}$ for 35 s, and extension at 72°C for 40–50 s; with a final extension at 72°C for 5–8 min. The purified amplified products were sequenced by Sangon Biotech on an ABI 3730 XL automatic sequencer (Applied Biosystems, USA). The sequences were analyzed and aligned with the analogous sequences of wild-type PBP2 in *N. gonorrhoeae* (LM306, GenBank accession no. M32091) by using BLAST.

The *N. gonorrhoeae* isolates with decreased susceptibility to CRO were genotyped by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) method, and the *PorB* and *tbpB* were amplified using primers and amplification parameters as previously described (Martin et al., 2004).

2.7. Data management and statistical analysis

The sensitivities and specificities of the in-house multiplex TaqMan real-time PCR platform with their corresponding 95% confidence intervals (CIs) were calculated using the statistical software SPSS version 22.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Antimicrobial resistance and NG-MAST analysis

Of the 897 *N. gonorrhoeae* isolates analyzed, 77 (8.58%) isolates exhibited decreased susceptibility to CRO (MICs: 0.125–0.50 mg/L) (Table S2),

Table 1

Target genes and mutations, primers/TaqMan probes, purpose and affected antibiotics, and multiplex combinations for the real-time PCR platform.

Target and mutations	Primers/TaqMan probes sequences	Amplicon lengths (bp)	Purpose and affected antibiotics	Multiplex type
<i>opa</i>	F (5'-AACGCATTACCCACGATTATCC-3') R (5'-TGTTTCTGCCATCATCGCTTACC-3') Probe (5'-AACCAACCGCTCCAGG-3')	98	NG species identification	Triplex 1
<i>porA</i>	F (5'-ATCCGACCCGCAATTGT-3') R (5'-TCCCGCATATCCGCTTCCT-3') Probe (5'-CAATCAGTCTCCAATTAC-3')	107	NG species identification	Triplex 1
<i>penA</i> G545S	F (5'-ACCGCGCAAGTTCGTC-3') R (5'-TATGGCTACCGCCACAATCAC-3') Probe (5'-TTATGCCTCCAACAAC-3')	106	Decreased susceptibility to CRO	Triplex 1
<i>penA</i> A501V	F (5'-CGAGGCAAAGGCATATTC-3') R (5'-TCTAGCGCCGACATCGAAAC-3') Probe A501V (5'-AATCGACCGTCGCGCA-3')	112	Decreased susceptibility to CRO	Triplex 2
<i>penA</i> A501T	F (5'-CGAGGCAAAGGCATATTC-3') R (5'-TCTAGCGCCGACATCGAAAC-3') Probe A501T (5'-ATCGACCACGCGGAG-3')	112	Decreased susceptibility to CRO	Triplex 2
<i>penA</i> A501P	F (5'-CGAGGCAAAGGCATATTC-3') R (5'-TCTAGCGCCGACATCGAAAC-3') Probe A501P (5'-ATTGCTACCTCGCGCGG-3')	112	Decreased susceptibility to CRO	Triplex 2

and the remaining 820 isolates showed full susceptibility to CRO (MICs: 0.004–0.063 mg/L). Among them, 8 isolates (0.89%) had CRO MICs that were greater than or equal to the CLSI MIC breakpoint of 0.25 mg/L (Table S2) and they were re-tested to confirm their phenotypes. Among the 77 isolates with decreased susceptibility to CRO, one showed susceptibility to CFM (MIC: 0.063 mg/L), and the other 76 isolates showed decreased susceptibility to CFM (MICs: 0.25–0.50 mg/L) (Table S2).

Among the 77 patients infected with *N. gonorrhoeae* with decreased susceptibility to CRO, 69 patients infected with *N. gonorrhoeae* (CRO MICs: 0.125 mg/L) were cured by CRO with increased dose of 500 mg per day. The other 8 patients infected with *N. gonorrhoeae* with CRO MICs greater than or equal to the CLSI MIC breakpoint of 0.25 mg/L (CRO MICs: 0.25–0.5 mg/L) were cured by cefoperazone/sulbactam with a dose of 1.5 g per day.

By NG-MAST, 77 isolates with decreased susceptibility to CRO were divided into 39 different NG-MAST STs. The most frequent STs were 17,547 (n = 4) and 15,143 (n = 4). Of the 39 different STs, 6 STs were represented by three isolates, 20 STs were represented by two isolates, and 11 STs were represented by one isolate. Of the 39 STs, 6 STs, which have not been previously described, are waiting for newly assigned by Curator of Web of NG-MAST (Table S3).

3.2. Validation of the multiplex TaqMan real-time PCR platform

All the *N. gonorrhoeae* control strains were correctly identified by the presence of the *opa* and/or *porA* genes by the multiplex TaqMan real time PCR. WHO reference strain L was correctly identified by the presence of the Ala501Val substitution in PBP2. TA0787 strain was correctly identified by the presence of the Gly545Ser substitution in PBP2, and the TA0011 strain was correctly identified by the presence of the Ala501Thr substitution in PBP2. F89 strain was correctly identified by the presence of both Gly545Ser and Ala501Pro substitutions. None of the Gly545Ser and Ala501Val/Thr/Pro substitutions were identified in PBP2 in WHO reference strains G, M, O, and ATCC 49226.

3.3. Detection limits of the Multiplex TaqMan real-time PCR

To assess the detection limits of the multiplex PCR platform, 10-fold serial dilutions of 10⁵ to 10 copies/mL gDNA of WHO L, F89, and TA0011 strains were tested 10 times for each by the platform. The detection limits of the multiplex PCR ranged from 10² to 10³ copies/mL gDNA (Table S4).

3.4. Cross-reactions with nongonococcal *Neisseria* Spp.

To assess the cross-reactions with nongonococcal *Neisseria* spp., a panel of 7 different nongonococcal *Neisseria* species including

3 *N. meningitidis* strains, 3 *N. mucosa* strains, 3 *N. subflava* strains, 3 *N. sicca* strains, 3 *N. lactamica* strains, 3 *N. flava* strains, and 2 *N. bacilliformis* strains were tested by the multiplex TaqMan real time PCR. In all the 20 strains of nongonococcal *Neisseria* species, none of the *opa* and *porA* genes, and Gly545Ser and Ala501Val/Thr/Pro substitutions were identified. This demonstrated that there were no cross-reactions with nongonococcal *Neisseria* spp. observed in the multiplex PCR platform (Table 2).

3.5. Detection of the cultured isolates

Culture-based antimicrobial susceptibility testing showed that 77 of 897 isolates were decreased susceptibility to CRO (MICs: 0.125–0.50 mg/L). To evaluate the multiplex TaqMan real-time PCR platform, these 77 isolates were detected by the multiplex platform. At the same time, the multiplex TaqMan PCR detected 100 isolates with full susceptibility to CRO (MICs: 0.004–0.032 mg/L) as the control. All the *N. gonorrhoeae* control strains were included in each run.

Overall, all the 77 cultured isolates of *N. gonorrhoeae* with decreased susceptibility to CRO were correctly identified by the multiplex real-time PCR platform. Meanwhile, in 95 of 100 cultured isolates of *N. gonorrhoeae* with full susceptibility to CRO, none of the Gly545Ser and Ala501Val/Thr/Pro substitutions in PBP2 were identified. Therefore, compared to standard GC diagnostic method-bacterial culture and culture-based antimicrobial susceptibility testing, the multiplex platform had a sensitivity of 100% (95% CI, 97.2–100%) and a specificity of 95.0% (95% CI, 89.9–99.7%) for identifying cultured isolates of *N. gonorrhoeae* with decreased susceptibility to CRO. In details, the multiplex real-time PCR not only correctly identified the cultured isolates as *N. gonorrhoeae* with 100% sensitivity and 100% specificity, but also offered sensitivities of 100% and specificities of 98.0–100% for identifying the mutations associated with decreased susceptibility to CRO from the cultured isolates (Table 2). Furthermore, the mutations in PBP2 associated with decreased susceptibility to CRO, identified by the multiplex real-time PCR, were confirmed by DNA sequencing (Table S2).

3.6. Direct detection of the clinical urogenital swabs

On the basis of culture-based MALDI-TOF MS and antimicrobial susceptibility testing and sequencing, 11 Gly545Ser substitutions, 51 Ala501Val substitutions, and 18 Ala501Thr substitutions were confirmed in the 77 *N. gonorrhoeae* positive clinical urogenital swabs containing the above-described mutations, associated with decreased susceptibility to CRO (Table 2).

Table 2
Performance of the multiplex TaqMan real time PCR platform in characterizing 77 *N. gonorrhoeae* isolates and their corresponding swabs from Shandong, China, during 2013–2017.

Purpose or affected antibiotic	Targets or mutations	Isolates or Swabs	NG with decreased susceptibility to CRO (n = 77; MICs: 0.125–0.5 mg/L)		NG with full susceptibility to CRO (n = 100; MICs: 0.004–0.032 mg/L)		Nongonococcal <i>Neisseria</i> species strains ^a (n = 20)		Sensitivity ^b (95% CI ^c)	Specificity ^b (95% CI ^c)
			Positive	Negative	Positive	Negative	Positive	Negative		
			Species identification	<i>opa</i> and/or <i>porA</i>	Isolates Swabs	77 77	0 0	100 100		
Decreased susceptibility to CRO	Gly545Ser	Isolates	11	66	1	99	0	20	100% (81.3–100%)	99.0% (86.2–100%)
		Swabs	11	66	1	99	0	20	100% (81.3–100%)	99.0% (86.2–100%)
	Ala501Val	Isolates	51	26	2	98	0	20	100% (95.8–100%)	98.0% (94.4–100%)
		Swabs	49	28	2	98	0	20	96.1% (89.1–99.6%) 98.0% (94.4–100%)	98.0% (94.4–100%)
	Ala501Thr	Isolates	18	59	2	98	0	20	100% (88.3–100%)	98.0% (94.4–100%)
		Swabs	17	60	2	98	0	20	94.4% (79.5–100%)	98.0% (94.4–100%)

^a The 20 nongonococcal *Neisseria* species strains were only used for calculating the specificity of the multiplex TaqMan real time PCR for species identification of *N. gonorrhoeae*.

^b Specificity was the probability that an isolate categorized as being sensitive to CRO was identified as negative by the multiplex real time PCR; sensitivity was the probability that an isolate categorized as showing decreased susceptibility to CRO was identified as positive by the multiplex real time PCR.

^c CI, confidence interval.

In the present study, the above-described 77 *N. gonorrhoeae* positive clinical urogenital swabs were directly detected by the multiplex real-time PCR platform. Simultaneously, the above-described 100 clinical urogenital swabs containing *N. gonorrhoeae* with full susceptibility to CRO were detected as the control. DNA was extracted from the clinical urogenital swabs and subsequently added to the multiplex PCR reaction mixture.

Overall, 74 of 77 above-described *N. gonorrhoeae* positive clinical urogenital swabs were correctly identified by the multiplex real-time PCR platform. Meanwhile, in 95 of 100 clinical urogenital swabs containing *N. gonorrhoeae* with full susceptibility to CRO, none of the Gly545Ser substitution and Ala501Val/Thr/Pro substitutions in PBP2 were identified. Therefore, the multiplex platform offered a sensitivity of 96.1% (95% CI, 90.7–99.2%) and a specificity of 95.0% (95% CI, 89.9–99.7%) for identifying *N. gonorrhoeae* with decreased susceptibility to CRO from clinical urogenital swabs. In details, the multiplex real-time PCR not only correctly identified *N. gonorrhoeae* in the clinical urogenital swabs with 100% sensitivity and 100% specificity, but also offered sensitivities of 94.4–100% and specificities of 98.0–100% for directly identifying the mutations associated with decreased susceptibility to CRO in *N. gonorrhoeae* from clinical urogenital swabs (Table 2).

4. Discussion

Currently, the *N. gonorrhoeae* isolates with decreased susceptibility and resistance to ESCs spread rapidly and seriously threaten the public health globally (Jiang et al., 2017; Papp et al., 2017; Peng et al., 2017). Treatment failures with CRO for gonorrhoea patients occur frequently due to infection of the *N. gonorrhoeae* with decreased susceptibility to CRO (Barbee, 2014; Gu et al., 2014; Katz et al., 2017). In this study, 8.58% (77/897) of *N. gonorrhoeae* isolates exhibited decreased susceptibility to CRO (MICs \geq 0.125 mg/L). Moreover, the most prevalent STs associated with decreased susceptibility to CRO in this district were 17,547 and 15,143, which were different from those reported in other districts in China (Chen et al., 2016; Liang et al., 2016; Jiang et al., 2017) and other countries in the world (Gianecini, et al., 2017; Papp et al., 2017; Peterson et al., 2015). The level (8.58%) of decreased susceptibility to CRO in this district was similar to those of three other cities in China reported: Hefei (11.1%, in 2014–2015) (Jiang et al., 2017), Changsha (11.9%, in 2015) (Peng et al., 2017) and Shanghai (7–13%, in 2008–2013) (Gu et al., 2014). However, it was much lower than that of Nanning city in China reported (32.9%, in 2000–2012, $P < 0.001$) (Zhu et al., 2014). It was also similar to one report in Hawaii in 2016, who said five of 61 (8.20%) *N. gonorrhoeae* isolates had MICs of 0.125–0.25 mg/L for ceftriaxone (Papp et al., 2017). In addition, it was

much lower than another report from USA CDC confirmatory laboratories who said five of eight *N. gonorrhoeae* isolates had MICs of 0.125 mg/L for ceftriaxone (Katz et al., 2017).

At present, a majority of clinical microbiology laboratories in China use CLSI MIC guidelines for interpreting the results of the antibiotic susceptibility tests. However, CLSI MIC guidelines have no resistance breakpoint for AZM against *N. gonorrhoeae*. Furthermore, in EUCAST MIC guidelines, the resistance breakpoint for CRO (MIC of >0.125 mg/L) against *N. gonorrhoeae* is lower than that (MIC of >0.25 mg/L) in CLSI guidelines. In general, the lower resistance breakpoint for CRO in EUCAST MIC guideline is chosen to be used for interpreting the MIC data for CRO. However, when CRO MICs are greater than or equal to the CLSI MIC breakpoint of 0.25 mg/L, the MIC data for CRO are also interpreted by the CLSI criteria.

The detection of *N. gonorrhoeae* with decreased susceptibility to CRO is very complicated since the mechanism of decreased susceptibility to CRO in *N. gonorrhoeae* is usually involved in multiple gene mutations (Peterson et al., 2015; Valentina et al., 2016). Latest studies have shown that the key mutations associated with decreased susceptibility to CRO concentrate on *penA* gene in *N. gonorrhoeae* (André et al., 2016; Bharat et al., 2015; Chen et al., 2016; Gianecini et al., 2017; Gong et al., 2016; Tomberg et al., 2017; Valentina et al., 2016). The detection limits of the in-house multiplex TaqMan real-time PCR platform were lower than those of a previous study (10^3 to 10^4 gDNA copies/reaction) (Valentina et al., 2016), ranging between 10^2 and 10^3 copies/mL. The multiplex platform had a very high sensitivity of 100% and a very high specificity of 95.0% for screening the *N. gonorrhoeae* with decreased susceptibility to CRO from the cultured isolates. Even when directly screening *N. gonorrhoeae* with decreased susceptibility to CRO from clinical urogenital swabs, the multiplex platform still had a high sensitivity of 96.1% and a high specificity of 95.0%. Only two swabs containing a single Ala501Val substitution in PBP2 and one swab containing a single Ala501Thr substitution in PBP2 in *N. gonorrhoeae* were not correctly identified, that might be due to very low loads of *N. gonorrhoeae* in these clinical samples (Valentina et al., 2016). Therefore, on the basis of sample culture and antimicrobial susceptibility testing in vitro, the in-house multiplex TaqMan real time PCR platform has been proven to be a sensitivity of 100% and a specificity of 95.0% useful tool for screening culture isolates of *N. gonorrhoeae* with decreased susceptibility to CRO, which can be finished within 2 days.

The current standard method used for GC diagnostic testing in China is bacterial culture and culture-based antimicrobial susceptibility testing. The current standard method in China takes approximately 3–4 days for confirming infections of *N. gonorrhoeae*. By the multiplex TaqMan real time PCR platform, the time taken for confirmation of *N. gonorrhoeae*

infections can be shortened to approximately 1 or 2 days. Therefore, the multiplex TaqMan real time PCR is a simple and rapid tool for confirming infections of *N. gonorrhoeae* with decreased susceptibility to CRO. Presently, PCR-based testing has been the gold standard test for *N. gonorrhoeae* and *Chlamydia* for many years in many developed countries in the world (Peterson et al., 2015, Vahidnia et al., 2015, Valentina et al., 2016). However, concurrent culture still should be encouraged, even though it takes longer time and lacks sensitivity, because generating isolates for antimicrobial susceptibility test is very important to understanding the resistance profiles of isolates circulating locally and regionally. In addition, patients infected with *N. gonorrhoeae* with decreased susceptibility to CRO should increase CRO dose from 250 to 500 mg like other areas in China (Chen et al., 2014, Gu et al., 2014, Zhu et al., 2014), or use spectinomycin (Zhao et al., 2018) or cefoperazone/sulbactam instead of using CRO. Meanwhile, due to the potential loss of effective and readily available treatment options, there is an immediate need to find out new alternative antimicrobial agents for treating *N. gonorrhoeae* infections (Singh et al., 2018, Zhao et al., 2018).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.diagmicrobio.2018.10.013>.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Competing interests

None declared.

Ethical Approval

Not required.

References

- André Z, Cécile M, Muhamed-Kheir T. Resistance to β -Lactams in *Neisseria* ssp due to chromosomally encoded penicillin-binding proteins. *Antibiotics* (Basel) 2016;5:35. <https://doi.org/10.3390/antibiotics5040035>.
- Barbee LA. Preparing for an era of untreatable gonorrhea. *Curr Opin Infect Dis* 2014;27:282–7. <https://doi.org/10.1097/QCO.0000000000000058>.
- Bharat A, Demczuk W, Martin I, Mulvey MR. Effect of variants of penicillin-binding protein 2 on cephalosporin and carbapenem susceptibilities in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2015;59:5003–6. <https://doi.org/10.1128/AAC.05143-14>.
- Breakpoint tables for interpretation of MICs and zone diameters: Version 7.1. European Committee on Antimicrobial Susceptibility Testing. Available from: http://www.eucast.org/clinical_breakpoints, 2017.
- Chen SC, Yin YP, Dai XQ, Unemo M, Chen XS. Antimicrobial resistance, genetic resistance determinants for ceftriaxone and molecular epidemiology of *Neisseria gonorrhoeae* isolates in Nanjing, China. *J Antimicrob Chemother* 2014;69:2959–65. <https://doi.org/10.1093/jac/dku245>.
- Chen SC, Yin YP, Dai XQ, Unemo M, Chen XS. First nationwide study regarding ceftriaxone resistance and molecular epidemiology of *Neisseria gonorrhoeae* in China. *J Antimicrob Chemother* 2016;71:92–9. <https://doi.org/10.1093/jac/dkv321>.
- Clinical and Laboratory Standards Institute (CLSI). *Neisseria gonorrhoeae*. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015. p. 80–2.

- Gianecini R, Romero MLM, Oviedo C, Vacchino M, Galarza P, Gonococcal Antimicrobial Susceptibility Surveillance Programme–Argentina (GASSP-AR) Working Group. Emergence and spread of *Neisseria gonorrhoeae* isolates with decreased susceptibility to extended-spectrum cephalosporins in Argentina, 2009 to 2013. *Sex Transm Dis* 2017;44:351–5. <https://doi.org/10.1097/OLQ.0000000000000603>.
- Gong Z, Lai W, Liu M, Hua Z, Sun Y, Xu Q, et al. Novel genes related to ceftriaxone resistance found among ceftriaxone-resistant *Neisseria gonorrhoeae* strains selected in vitro. *Antimicrob Agents Chemother* 2016;60:2043–51. <https://doi.org/10.1128/AAC.00149-15>.
- Gu WM, Chen Y, Yang Y, Wu L, Hu WZ, Jin YL. Twenty-five-year changing pattern of gonococcal antimicrobial susceptibility in Shanghai: surveillance and its impact on treatment guidelines. *BMC Infect Dis* 2014;14:731. <https://doi.org/10.1186/s12879-014-0731-9>.
- Jiang FX, Lan Q, Le WJ, Su XH. Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates from Hefei (2014–2015): genetic characteristics of antimicrobial resistance. *BMC Infect Dis* 2017;17:366. <https://doi.org/10.1186/s12879-017-2472-z>.
- Katz AR, Komeya AY, Kirkcaldy RD, Whelen AC, Soge OO, PappJR, et al. Cluster of *Neisseria gonorrhoeae* isolates with high-level azithromycin resistance and decreased ceftriaxone susceptibility, Hawaii, 2016. *Clin Infect Dis* 2017;65:918–23. <https://doi.org/10.1093/cid/cix485>.
- Kumari R, Banerjee T, Anupurba S. Molecular detection of drug resistance to ofloxacin and kanamycin in *Mycobacterium tuberculosis* by using multiplex allele-specific PCR. *J Infect Public Health* 2018;11:54–8. <https://doi.org/10.1016/j.jiph.2017.03.007>.
- Liang JY, Cao WL, Li XD, Bi C, Yang RD, Liang YH, et al. Azithromycin-resistant *Neisseria gonorrhoeae* isolates in Guangzhou, China (2009–2013): coevolution with decreased susceptibilities to ceftriaxone and genetic characteristics. *BMC Infect Dis* 2016;16:152. <https://doi.org/10.1186/s12879-016-1469-3>.
- Martin IM, Ison CA, Aanensen DM, Fenton KA, Spratt BG. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. *J Infect Dis* 2004;189:1497–505. <https://doi.org/10.1086/383047>.
- Nakayama S, Shimuta K, Furubayashi K, Kawahata T, Unemo M, Ohnishi M. New ceftriaxone- and multidrug-resistant *Neisseria gonorrhoeae* strain with a novel mosaic penA gene isolated in Japan. *Antimicrob Agents Chemother* 2016;60:4339–41. <https://doi.org/10.1128/AAC.00504-16>.
- PappJR, Abrams AJ, Nash E, Katz AR, Kirkcaldy RD, O'Connor NP, et al. Azithromycin resistance and decreased ceftriaxone susceptibility in *Neisseria gonorrhoeae*, Hawaii, USA. *Emerg Infect Dis* 2017;23:830–2. <https://doi.org/10.3201/eid2305.170088>.
- Peng T, Lin H, Liu Q, Cao W, Ding H, Chen J, et al. Ceftriaxone susceptibility and molecular characteristics of *Neisseria gonorrhoeae* isolates in Changsha, China. *J Infect Chemother* 2017;23:385–9. <https://doi.org/10.1016/j.jiac.2017.03.007>.
- Peterson SW, Martin I, Demczuk W, Bharat A, Hoang L, Wylie J, et al. Molecular assay for detection of genetic markers associated with decreased susceptibility to Cephalosporins in *Neisseria gonorrhoeae*. *J Clin Microbiol* 2015;53:2042–8. <https://doi.org/10.1128/JCM.00493-15>.
- Severin G, Duylinh N, Daniella L, Samuel M, Bauer H, Pandori M. *Neisseria gonorrhoeae* and extended-spectrum cephalosporins in California: surveillance and molecular detection of mosaic penA. *BMC Infect Dis* 2013;13:570. <https://doi.org/10.1186/1471-2334-13-570>.
- Singh V, Bala M, Bhargava A, Kakran M, Bhatnagar R. In vitro efficacy of 21 dual antimicrobial combinations comprising novel and currently recommended combinations for treatment of drug resistant gonorrhoea in future era. *PLoS One* 2018;13, e0193678. <https://doi.org/10.1371/journal.pone.0193678>.
- Tabrizi SN, Su J, Bradshaw CS, Fairley CK, Walker S, Tan LY, et al. Prospective evaluation of Resistance Plus MG, a new multiplex quantitative PCR assay for detection of *Mycoplasma genitalium* and macrolide resistance. *J Clin Microbiol* 2017;55:1915–9. <https://doi.org/10.1128/JCM.02312-16>.
- Takahata S, Senju N, Osaki Y, Yoshida T, Ida T. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2006;50:3638–45. <https://doi.org/10.1128/AAC.00626-06>.
- Thakur SD, Araya P, Borthagaray G, Galarza P, Hernandez AL, Payares D, et al. Resistance to ceftriaxone and azithromycin in *Neisseria gonorrhoeae* isolates from 7 countries of South America and the Caribbean: 2010–2011. *Sex Transm Dis* 2017;44:157–60. <https://doi.org/10.1097/OLQ.0000000000000587>.
- Tomberg J, Fedarovich A, Vincent LR, Jerse AE, Unemo M, Davies C, et al. Alanine501 mutations in penicillin-binding protein 2 from *Neisseria gonorrhoeae*: structure, mechanism, and effect on cephalosporin resistance and biological fitness. *Biochemistry* 2017;56:1140–50. <https://doi.org/10.1021/acs.biochem.6b01030>.
- Unemo M, Fåth O, Fredlund H, Limnios A, Tapsall J. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. *J Antimicrob Chemother* 2009;63:1142–51. <https://doi.org/10.1093/jac/dkp098>.
- Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. *Clin Microbiol Rev* 2014;27:587–613. <https://doi.org/10.1128/CMR.00010-14>.
- Vahidnia A, van Empel PJ, Costa S, Oud RT, van der Straaten T, Blikendaal H, et al. A misleading false-negative result using *Neisseria gonorrhoeae* opa MGB multiplex PCR assay in patient's rectal sample due to partial mutations of the opa gene. *New Microbiol* 2015;38:431–4.
- Valentina D, Sara K, Agnese L, Guilarte YN, Hauser C, Furrer H, et al. Multiplex real-time PCR assay with high-resolution melting analysis for characterization of antimicrobial resistance in *Neisseria gonorrhoeae*. *J Clin Microbiol* 2016;54:2074–81. <https://doi.org/10.1128/JCM.03354-15>.
- Whiley DM, Goire N, Lambert SB, Ray S, Limnios EA, Nissen MD, et al. Reduced susceptibility to ceftriaxone in *Neisseria gonorrhoeae* is associated

- with mutations G542S, P551S and P551L in the gonococcal penicillin-binding protein 2. *J Antimicrob Chemother* 2010;65:1615–8. <https://doi.org/10.1093/jac/dkq187>.
- World Health Organization (WHO). Global action plan to control the spread and impact of anti-microbial resistance in *Neisseria gonorrhoeae*. Geneva: WHO; 2012. Available from: http://apps.who.int/iris/bitstream/10665/44863/1/9789241503501_eng.pdf.
- Zhao L, Liu A, Li R, Zhao S. Trends in antimicrobial resistance in *Neisseria gonorrhoeae* and molecular characteristics of *Neisseria gonorrhoeae* with decreased susceptibility to ceftriaxone in Shandong, China, 2007 to 2014. *Int J Antimicrob Agents* 2018;51:52–6. <https://doi.org/10.1016/j.ijantimicag.2017.06.004>.
- Zhu BY, Yu RX, Yin Y, Chen X, Li W, Dai XQ, et al. Surveillance of antimicrobial susceptibilities of *Neisseria gonorrhoeae* in Nanning, China, 2000–2012. *Sex Transm Dis* 2014;41:501–6. <https://doi.org/10.1097/OLQ.0000000000000157>.