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Short Communication

A real-time PCR assay for detecting a *penA* mutation associated with ceftriaxone resistance in *Neisseria gonorrhoeae*

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ABSTRACT

Objectives: Ceftriaxone (CRO) resistance is spreading worldwide, and hindering the effective treatment of gonococcal infections. This study developed a detection system for the genomic DNA of CRO-resistant *Neisseria gonorrhoeae* (*N. gonorrhoeae*) strains, in order to improve the surveillance of antimicrobial resistance.

Methods: A real-time PCR assay targeting the *penA* gene of recently isolated CRO-resistant *N. gonorrhoeae* strains was designed. Primer and probe sequence information was obtained from sequence comparisons between *penA* of *Neisseria* spp. and *penA* of CRO-resistant *N. gonorrhoeae* strains.

Results: Using this assay, a positive reaction was observed using the genomic DNA of three strains (GU140106, FC428, and A8806). The assay was evaluated using genomic DNA of 204 *N. gonorrhoeae* and 95 *Neisseria* spp. isolates with known minimum inhibitory concentrations of CRO. Following PCR assays for these strains, three FC428-related strains were positively identified, which possessed *penA*-60.001, whereas the remaining 201 *N. gonorrhoeae* strains and 95 *Neisseria* spp. strains were negative.

Conclusions: A real-time PCR-based assay was designed to detect the genomic DNA of strains harbouring mosaic *penA*-59.001 (GU140106), *penA*-60.001 (FC428), and *penA*-64.001 (A8806) alleles and to discriminate them from *N. gonorrhoeae* and *Neisseria* spp. strains harbouring other genes.

1. Introduction

In recent years, the multidrug resistance of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) has shown an increasing trend [1]. Sensitivity of *N. gonorrhoeae* to extended-spectrum cephalosporins (ESCs) is decreasing [1]. One ESC, ceftriaxone (CRO), is usually recommended as the first-line agent for the treatment of infections caused by gonococci [1]; however, due to the development of CRO resistance, this antibiotic is not as effective as it should be in some cases. Mutations in the *penA* gene, which encodes the penicillin-binding protein 2 (PBP2), may cause resistance [2]. This gene has become a mosaic gene by acquiring genomic DNA from *Neisseria* spp. through natural transformation [3]. Amino acid changes in PBP2 at positions 311 (alanine to valine), 316 (valine to proline), and 483 (threonine to serine) have increased the minimum inhibitory concentration (MIC) of CRO [2]. In recent years, mutations of A311V and T483S, but not V316P, have been frequently observed in PBP2 of CRO-resistant gonococci, including strains GU140106, FC428, and A8806 [4–10]. These strains harbour mosaic *penA*-59.001 (GU140106), *penA*-60.001 (FC428), and *penA*-64.001 (A8806; WHO-Z) alleles [4–6].

Because of the serious spread of CRO resistance around the world, it is imperative that simple and accurate methods be developed to reliably detect CRO-resistant gonococci [4–10]. Therefore, this study attempted to develop a system to detect CRO-resistant gonococcal genomic genes harbouring *penA*-59.001, *penA*-60.001, and *penA*-64.001. A real-time PCR assay for detecting three types of *penA* genes in *N. gonorrhoeae* was proposed, and this

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assay may be adopted to improve the surveillance of antimicrobial resistance in *N. gonorrhoeae*.

2. Materials and methods

2.1. Neisseria *spp. and antimicrobial susceptibility*

This study obtained 204 isolates of *N. gonorrhoeae* in Japan in 2015. The detailed information about these strains has previously been reported [11]. The GU140106 and AM1601 strains were obtained from Gifu University and Aichi Medical University in Japan, respectively [5,12]. From 2011 to 2016, 95 *Neisseria* spp. strains were isolated during routine examinations for sexually transmitted infections (gonococcus-negative pharyngeal specimens). Antimicrobial susceptibility was determined using Etest (bioMérieux, Marcy-l'Étoile, France), as previously described [13]. The species of all *Neisseria* spp. strains were verified using 53 ribosomal protein subunit genes, as previously described [12,14].

2.2. Genomic DNA purification

Genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). To accurately determine the double-stranded DNA concentration, the Qubit$^{\text{TM}}$ dsDNA HS Assay Kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) were used. The same purified genomic DNA was used for whole-genome sequencing (WGS) and real-time PCR assay.

2.3. World Health Organization reference gonococcal strains

The genomic DNA of World Health Organization (WHO) strains was kindly gifted by Dr Unemo Magnus of Örebro University in Sweden [15]. The *penA* sequences were extracted from each genome sequence: WHO-F (GenBank accession number LT591897.1), WHO-G (GenBank accession number LT591898.1), WHO-K (GenBank accession number LT591908.1), WHO-L (GenBank accession number LT591901.1), WHO-M (GenBank accession number LT591904.1), WHO-N (GenBank accession number LT591910.1), WHO-O (GenBank accession number LT592146.1), WHO-P (GenBank accession number LT592157.1), WHO-U (GenBank accession number LT592159.1), WHO-V (GenBank accession number LT592150.1), WHO-W (GenBank accession number LT592163.1), WHO-X (GenBank accession number LT592155.1), WHO-Y (GenBank accession number LT592161.1), and WHO-Z (GenBank accession number LT592153.1).

2.4. Next-generation sequencing

To obtain WGS information, the MiSeq platform (Illumina, San Diego, CA, USA) was used. Genomic DNA libraries for WGS were prepared using a Nextera XT DNA sample prep kit (Illumina). The pooled libraries were subjected to multiplexed paired-end sequencing (300 mer × 2). The assembled sequence was generated using CLC Genomics Workbench 8.5.1 (Qiagen).

2.5. penA *sequence comparison*

The *penA* alleles from SI28-1, SI60-1, SI57-1, and SI94-3 were extracted from WGS data using FA1090 as a reference sequence, and the absence of misreads was subsequently confirmed by the conventional Sanger sequencing method, as previously described [12]. The complete nucleotide sequences of the *penA* gene of SI28-1, SI60-1, SI57-1, and SI94-3 have been deposited in the DNA Data Bank of Japan under accession numbers **LC410041**–**LC410044**. The previously published *penA* sequences (GenBank: AB904142, AB904147, AB904125.1, AB904126.1, LC316656.1) of *Neisseria* spp. SH43-1, SH43-3, NLA-1, NSU-per-57, and AM1601, respectively, were used. Sequences were compared in multiple-sequence alignment using Clustal W. The *penA* sequence of *N. gonorrhoeae* was assigned using the NG sequence typing for antimicrobial resistance (NG-STAR) [16].

2.6. Real-time PCR assay design and conditions

By sequence comparisons between various types of *penA* alleles of *N. gonorrhoeae* and *penA* of *Neisseria* spp. using Clustal W, a specific region of *penA*-59.001 (GenBank: LC056026.1) and *penA*-60.001 (GenBank: LC113953.1) alleles was identified. It has previously been reported that the 3′-terminal half region of *penA*-59.001 and *penA*-60.001 exhibits a high similarity with *penA* of a *Neisseria* spp. strain, which shows a high ESC MIC [12]. Therefore, to suppress amplification with gonococcus *penA*, other than *penA*-59.001 and *penA*-60.001, a reverse primer was designed from sequences in the 3′-terminal half region of these *penA* sequences. To determine the specific sequences of *Neisseria* spp. strains, some strains from routine examinations for sexually transmitted infections were obtained using gonococcus-negative pharyngeal specimens as well as the AM1601 and SH43-3 strains, which have been previously reported [12]. To determine unique *N. gonorrhoeae penA* sequences, the *penA* sequences of the WHO reference strains exhibiting various CRO MIC ranges were used [15]. The 14 reference strains possess various types of *penA*. The forward primer was designed from sequences in the 5′-terminal half region of *penA*-59.001 and *penA*-60.001. As a result, to specifically amplify *penA*-59.001 and *penA*-60.001, forward and reverse primers were designed from sequences in the 5′-terminal half region and the 3′-terminal half region of *penA*-59.001 and *penA*-60.001, which are unique and do not completely overlap with those found in *N. gonorrhoeae penA* and *Neisseria* spp. *penA*. Based on this analysis, the forward primer was selected at nucleotide position 198–210 and the reverse primer at nucleotide position 981–1001 from the start codon of *penA*-60.001 (Table 1). A TaqMan probe was used for fluorescent mutation detection of nucleotide positions 921–943 from the start codon of the gene. In addition, as shown in Fig. 1, the primers and probe sequences matched those of *penA*-64.001, corresponding to WHOZ.

The PCR mixture contained 12.5 μL of QuantiTect Probe PCR Master Mix (Qiagen), 10.0 pmol of each forward and reverse primer, 4.0 pmol of the TaqMan FC428 probe, and 2.5 μL of various prepared solutions (genomic DNA or bacterial culture) for the template, with a final reaction volume of 25 μL. The PCR conditions were as follows: initial denaturation at 95 °C for 15 min, 45 cycles at 95 °C for 5 s, and 60 °C for 60 s. Amplification and signal detection were performed using a 7500 Real-Time PCR System instrument (Applied Biosystems, Foster City, CA, USA), and melting curves were analysed using 7500 System SDS software version 1.4 (according to the manufacturer's instructions).

3. Results and discussion

The purpose of this study was to develop a simple and specific assay with which to reliably detect the *penA* gene of CRO-resistant

Table 1
Primer and probe sequences.

	Target sequence (5′–3′)
Forward	CCCCGCGGACTCAAGCA
Reverse	GTATTGAATGTGTCTGTTGGA
Probe	FAM-ACCTGGTTCTGTCATGAAGCCGT-TAMRA

Note: To match the melting temperature (Tm) of the reverse primer, a mismatch sequence shown as CCCC was introduced into the 5′-terminal end of the forward primer.

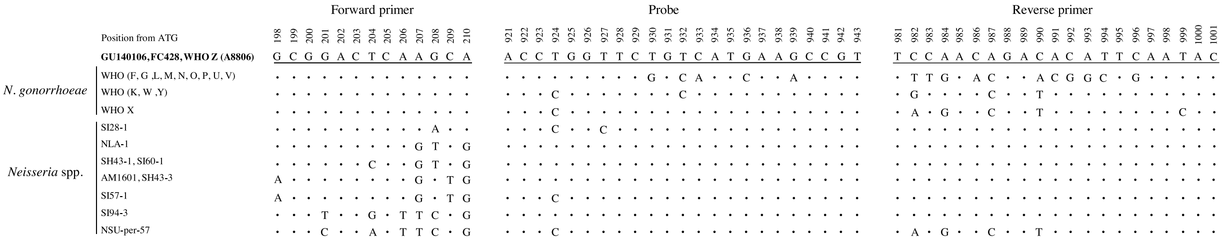

Fig. 1. Sequence alignment of the *penA* region used for primer and probe selection.

N. gonorrhoeae strain. First, a real-time PCR was performed with 10 ng of genomic DNA from WHO gonococcal reference strains (F, G, K, L, M, N, O, P, U, V, W, X, Y, Z), FC428 strain, and GU140106 strain. As a result, a positive signal was obtained only when GU140106, FC428, and WHOZ genomic DNA were used.

As shown in Fig. 1, the primer and probe sequences used in this assay were identical to the *penA* sequences found in *penA*59.001 (GU140106), *penA*60.001 (FC428), and *penA*64.001 (WHOZ; A8806). On the other hand, the *penA* sequences of WHO strains, except that of WHOZ strain, were not identical to the primer and probe sequences. Positive signals were observed with cycle threshold (C_t) values during cycles 21.2 and 23.8, whereas no positive signals were observed during cycle 45 when WHO genomic DNA, except that of WHOZ, were used. These results showed no discrepancy with primer and probe sequences.

To determine the detection limit of this assay, a range of genomic DNAs (one genome copy to 10^6 genome copies) were tested from the GU140106, FC428, and WHOZ (A8806) strains. It was found that significant signals were detected between 10 and 10^6 genome copies per reaction. These significant signals were observed with C_t values during cycles 22.9 and 43.1. In addition, the detection limit was determined using a dilution series of cultured FC428 and GU140106 strains ranging from 10^0–10^6 CFU/mL per reaction. It was determined that an optical density (OD_{600}) of 0.4 corresponds to a bacterial density of approximately 1.0×10^8 CFU/mL. It was found that reliable signals were shown between 10^2 (or 10^3) and 10^6 CFU/mL per reaction in FC428 and GU140106 strains, respectively.

Next, the sensitivity and specificity of the assay were evaluated using genomic DNA (10 ng/reaction) of 204 *N. gonorrhoeae* strains with known drug susceptibility [11]. Three strains (including the FC428 strain) exhibiting CRO MIC values $\geq$0.5 mg/L were positively identified, while all the 201 strains showing CRO MIC values <0.5 mg/L were negative. It was found that the *penA* sequence of the two strains (other than that of the FC428 strain) exhibiting CRO MIC values $\geq$0.5 mg/L was identical to the *penA* sequence of FC428 (Table 2) [11]. In addition, some strains harbouring *penA*-10.001 and *penA*-34.001 were found in these isolates, but were negative in the current assay. These *penA* types are often found in *N. gonorrhoeae* strains with reduced susceptibility to cephalosporins [13]. Based on these findings, the current assay had a high sensitivity (100%) and specificity (100%) for identifying the genomic DNA of strains FC428, GU140106, and A8806 (WHOZ).

Table 2
List of *Neisseria* spp. strains.

CRO MIC (mg/L)	≥0.5		<0.5	
PCR results	Positive	Negative	Positive	Negative
N. gonorrhoeae (n = 204)	3	0	0	201
N. mucosa var. heidelbergensis (n = 2)	0	0	0	2
N. mucosa (n = 28)	0	0	0	28
N. polysaccharea polyphyletic (n = 3)	0	2	0	1
N. subflava (n = 53)	0	2	0	51
N. cinerea (n = 6)	0	3	0	3
N. lactamica (n = 2)	0	2	0	0
N. meningitidis (n = 1)	0	0	0	1

Abbreviations: CRO, ceftriaxone; MIC, minimum inhibitory concentration.
Note: The strains with a CRO MIC value ≥0.5 mg/mL are *N. gonorrhoeae* (FC428, FC460, FC498), *N. polysaccharea* polyphyletic (SH43-1, SI28-1), *N. subflava* (NSU-per-57, SI94-3), *N. cinerea* (SH43-3, SI57-1, AM1601), and *N. lactamica* (NLA-1, SI60-1).

The *penA* allele of *N. gonorrhoeae and Neisseria* spp. could be cross-reacted in real-time PCR assays because *Neisseria* spp. *penA* and *N. gonorrhoeae* mosaic *penA* share regions with high similarity [12]. To investigate cross-reactivity of the *penA* allele of *Neisseria* spp. and that of *N. gonorrhoeae*, further experiments were performed. Using the real-time PCR assay and custom-designed primers and probes, 95 strains of *Neisseria* spp. were screened, all of which were negative. The CRO MIC values of these strains were confirmed; nine exhibited MIC values of $\geq$0.5 mg/L. By sequence analysis using Clustal W, it was found that the *penA* gene of the nine *Neisseria* strains with high ESC MICs has a sequence similarity of 82.3–94.5% with *penA*-59.001, 77.8–95.3% with *penA*-60.001, and 77.8–95.6% with *penA*-64.001. Furthermore, the *penA* gene of these nine *Neisseria* spp. strains encodes alterations including A311V and T483S, but not V316P. As shown in Fig. 1, a sequence alignment of the partial *penA* sequences of these *Neisseria* spp. strains did not completely overlap with the primers and probe sequence region.

Since the emergence of the first CRO-resistant strain – H041 – in 2009, additional resistant *N. gonorrhoeae* strains – F89, A8806, GU140106, and F428 – have been identified [4–6,17,18]. Fortunately, A8806, GU140106, and H041 strains have not been reported since their initial appearance [13]. Similarly, the F89 strain was reported only once [19]. However, the CRO-resistant FC428-related strain has spread worldwide since its initial detection in 2015 [7–10]. As a result, there is a critical need to quickly and accurately identify such CRO-resistant gonococcal strains. To address this issue, the current study constructed a detection system using a real-time PCR targeting three types of *penA*, including *penA*-60.001 (FC428), *penA*-59.001 (GU140106), and *penA*-64.001 (A8806). As demonstrated here, the assay specifically detects genes of CRO-resistant *N. gonorrhoeae*; it does not detect other genes of *N. gonorrhoeae* strains or *Neisseria* spp.

The nucleic acid amplification test (NAAT) is commonly used to detect gonococcal genes. However, genes from *Neisseria* spp. often give false-positive reactions in NAAT for *N. gonorrhoeae* from pharyngeal specimens because *N. gonorrhoeae* is among the *Neisseria* spp. found in the pharynx [1]. Thus, to avoid false-positive reactions in the examination of *N. gonorrhoeae* isolated from pharyngeal specimens, it is important to ensure that the assay does not erroneously detect *Neisseria* spp. genes. Recently, a real-time PCR system that can detect *penA*-60.001 has been reported [20]. In that assay, *penA*-64.001 (A8806) was shown to produce a positive reaction, although its sequence mismatched with that of *penA*-60.001 (FC428) in terms of the forward primer. The *penA*-64.001 (A8806) sequence corresponded to the probe and each primer sequence exactly coincided with those of the *penA* of some commensal *Neisseria* spp. (SI 60-1, SH 43-1, NLA-1, SH 43-3, and SI 94-3) shown. This indicates that the genomic DNA of these commensal *Neisseria* spp. show a positive reaction similar to *penA*-64.001 (A8806), suggesting a possibility that a false-positive reaction may potentially occur in the above assay, as observed with *N. lactamica* [20]. On the other hand, the 95 commensal isolates of *Neisseria* spp. (some strains exhibit CRO MIC values of ≥0.5 mg/L) showed no positive reaction, even during cycle 45 in the current assay; thus, this assay could be used to detect genes of CRO-resistant *N. gonorrhoeae* from pharyngeal specimens.

This study described an assay that accurately detects a genomic gene of CRO-resistant gonococci. Since the spread of CRO-resistant strains is a major public health concern, it is important to identify these strains early, before the infection spreads. Although further validation of the assay using clinical specimens is required before it can be applied in the clinical setting, it is hoped that this simple, accurate, and robust test will contribute to antimicrobial resistance surveillance of *N. gonorrhoeae*.

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Competing interests

None.

Ethical approval

Not required.

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