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SUSCEPTIBILITY

A Multiplex Molecular Assay for Detection of Six *penA* Codons To Predict Decreased Susceptibility to Cephalosporins in *Neisseria gonorrhoeae*

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ABSTRACT The emerging cephalosporin-resistant *Neisseria gonorrhoeae* poses an urgent threat to the continued efficacy of the last-line monotherapy for gonorrhea. Consequently, high-throughput, accurate, and reasonable molecular assays are urgently needed for strengthening antimicrobial-resistance surveillance in *N. gonorrhoeae*. In this study, we designed a high-throughput multiplex method that incorporates high-resolution melting technology and is based on a 6-codon assay (among the most parsimonious assays) developed following comprehensive and systematic reviews. The results showed that our method can precisely distinguish specific single-nucleotide polymorphisms in resistance-associated genes with a specificity and sensitivity of 100% and a detection limit as low as 10 copies per reaction. This method can be directly applied to clinical samples without cumbersome culture and successfully predicted all cephalosporin-resistant isolates (sensitivity: 100%). The method presented here represents a technique for rapid testing of antimicrobial resistance and will serve as a valuable tool for tailor-made antimicrobial therapy and for monitoring the transmission of cephalosporin-resistant strains.

KEYWORDS antimicrobial resistance, cephalosporin, HRM, molecular assay, *Neisseria gonorrhoeae*, 6-codon

Gonorrhea caused by *Neisseria gonorrhoeae* was the second most common sexually transmitted bacterial infection (STI) in 2020, with an estimated 82.4 million new cases worldwide (1). Antimicrobial resistance (AMR) significantly threatens the prevention and control of gonorrhea, which remains a global health concern (2, 3). Due to the increased resistance to azithromycin in other STI infections, the latest guidelines from the United States Centers for Disease Control and Prevention state that traditional dual therapy (mainly azithromycin and cephalosporin; CEP) should be replaced by monotherapy (ceftriaxone; CRO). Additionally, the CRO dose should be doubled (250 mg → 500 mg) according to CRO pharmacokinetics data. This indicates that the benefits of maintaining dual therapy suggested by the World Health Organization have gradually decreased in some areas (4). Unfortunately, in recent years, increasing trends of CEP-resistant isolates have been reported globally, indicating that the "last-line" therapy faces the threat of untreatable gonorrhea (5–8), thereby highlighting the necessity to enhance AMR surveillance of *N. gonorrhoeae* (9).

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The culture method has long been established in determining antimicrobial susceptibility; however, long culture times and cumbersome hands-on processes limit the number of samples monitored, making the scale of annual monitoring far smaller than that required for the analysis of new cases (9). Consequently, nucleic acid amplification tests (NAATs) are widely used in many well-resourced settings owing to their high-throughput and multiplex format (5, 10, 11). In recent decades, various molecular markers serving as assay targets have been used in NAATs to detect resistant strains. Notably, the *penA* gene encodes penicillin-binding protein 2, a major lethal target for CEPs. The mosaic structure of the *penA* gene was produced by DNA transformation with nongonococcal *Neisseria* spp., which represent the most common target of CEP resistance. Compared with the wild-type *penA* gene, the mosaic *penA* gene contains substantial amino acid alterations in the transpeptidase domain (residues 263–557) (Fig. S1 in the supplemental material) (12, 13). Additionally, mosaic *penA* provides a potential assay target for most existing molecular assays of CEP resistance, including real-time PCR, loop-mediated isothermal amplification (14), mass spectrometry, and several sequencing-based methods (15–17).

A recent systematic review of all reported *N. gonorrhoeae* isolates ($n = 415$) from 22 countries (from January 1995 to November 2018) with decreased susceptibility to cefixime (the most potent oral CEP) found that 99.5% of all isolates harbored a mosaic *penA* (identified by targeting a unique structure at position 375–377) or at least one alteration at positions 501, 542, and 551 in nonmosaic *penA* alleles (18). Similarly, another study analyzed the genetic characterization of 3,821 *N. gonorrhoeae* isolates from 23 countries (as of October 2019) with known MICs of CRO. The strategy of detecting at least one of the three alterations at positions 501, 542, and 551 in nonmosaic *penA* strains showed better performance than other combinations of AMR sites (95% sensitivity and 62% specificity) (5). Among nonmosaic *penA* isolates, previous reports offer substantial evidence of the correlation between mutations at positions 501/542/551 (especially P551S/L and G542S) and decreased susceptibility to CEP (CEP-DS) (19). In the present study, we established a rapid molecular tool based on high-resolution melting (HRM) technology to scan known 6-codon targets (amino acid positions 375–377, 501, 542, and 551; derived from previous global comprehensive analyses) that indicate reduced susceptibility to antimicrobial agents in *N. gonorrhoeae* (5, 18). Therefore, the goal of this study was to design a multiplex HRM assay to predict decreased susceptibility to CEP in *N. gonorrhoeae* in order to precisely guide personal treatment and monitor resistance phenotypes without time-consuming culture procedures. We further evaluated the ability of this HRM assay to predict decreased susceptibility to CEP (CRO and cefixime) according to different decision strategies.

RESULTS

HRM assay. In this study, we devised an HRM analysis method that targets six codons in *penA* and is developed based on a comprehensive systematic review of the molecular characteristics of a large number of international CEP-DS *N. gonorrhoeae* isolates, which comprised two triplex assays (targeting mosaicism or the A501mut, G542mut, and P551mut in nonmosaic isolates) (Table 1). The results showed that the primer for targets 375 through 377 could accurately screen nine important mosaic *penA* types, including *penA*-60.001, which is currently spreading internationally, and the common resistant type *penA*-34.001, suggesting these positions as reliable targets for distinguishing all mosaic *penA* types from the nonmosaic structure. Additionally, we found that targets 501, 542, and 551 could only provide effective data in nonmosaic patterns. These results indicated that the 6-codon method could correctly distinguish specific amino acid mutations from the wild type (Fig. 2), particularly positions 501 and 542, where the temperature difference of the melting curve is >1°C. The melting temperature (Tm) ranges of all the targets are listed in Table 2.

We then collected a total of 346 isolates with known AMR profiles based on Sanger sequencing to evaluate the effectiveness of the 6-codon method. The data showed that this method accurately detected all common *penA* types and demonstrated

TABLE 1 Optimal primers of two triple assays used in the 6-codon method

Assay	Target gene	Primer sequence	Primer concn (μM)	Significance	Reference
Assay1	*Opa*	opa_F: CCGGCGGCGTTCAGCACCTTAGGGAACCG	0.125	Species identification	(21)
		opa_R: CGGCGGCCGTGGCGGATGAACAAAAACCG	0.125		
	penA	PenA_375-377_F: ACGTCGGTACCAGTAAACTTTCTGC	0.25	Predict decreased susceptibility to cephalosporin in mosaic type	This study
		PenA_375-377_R: CGTGATAGAAATCGTACATTTCTTT	0.25		
	penA	PenA_A501_F: GATGTCGGCGCTAAAACC	0.75	Predict decreased susceptibility to cephalosporin in nonmosaic type	This study
		PenA_A501_R: AGCGGCCATTGACCAGTT	0.75		
Assay2	*penA*	PenA_G542 _F: CGACGAACCGACTGCCCAC	0.5		This study
		PenA_G542 _R: GCCACTACGCCGCCGTAATAG	0.5		
		PenA_P551 _R: GCCCATAATTTTTTTGAAGGG	0.5		
	porA	porA_F: CCTGCTACTTTCACGCTGGA	0.25	Species identification	This study
		porA_R: CCGGAACTGGTTTCATCTGA	0.25		

considerable consistency with Sanger sequencing. In particular, we found a perfect association (100%) between this method and Sanger sequencing at positions 375 through 377, 501, and 542 located in *penA*, with the remaining position (PenA_P551) also showing high concordance (specificity: 100%; sensitivity: 99.1%). Additionally, all 346 isolates were double-positive (both *opa* and *porA*), which confirmed the validity of these two genes for species identification (Table 3).

Cross-reactivity and detection limit of the 6-codon method. All 11 nongonococcal *Neisseria* spp. isolates were negative for both *opa* and *porA*, except for one *N. meningitidis* isolate that showed cross-reactivity with *opa*. Therefore, the results were accepted as valid when the isolates were positive for both *opa* and *porA*. Additionally, the 6-codon method was sensitive enough to determine the isolates with 10 copies per reaction for all targets.

Performance of the 6-codon method with clinical samples. We then collected a total of 153 *N. gonorrhoeae*-positive samples (confirmed by real-time PCR) to evaluate the detection limit of the 6-codon method. The DNA load of the urine samples was apparently lower than that of the urethral swabs. The distribution of the different concentrations of all 153 samples is shown in Table S4. For the urethral swabs (n = 141), 2/141 (1.42%) and 1/141 (0.71%) of samples failed to provide stable data in assays 1 and 2, respectively, which might be due to interference from other bacterial species. By contrast, all urine samples (n = 12) were successfully analyzed and showed stable performance at various concentrations

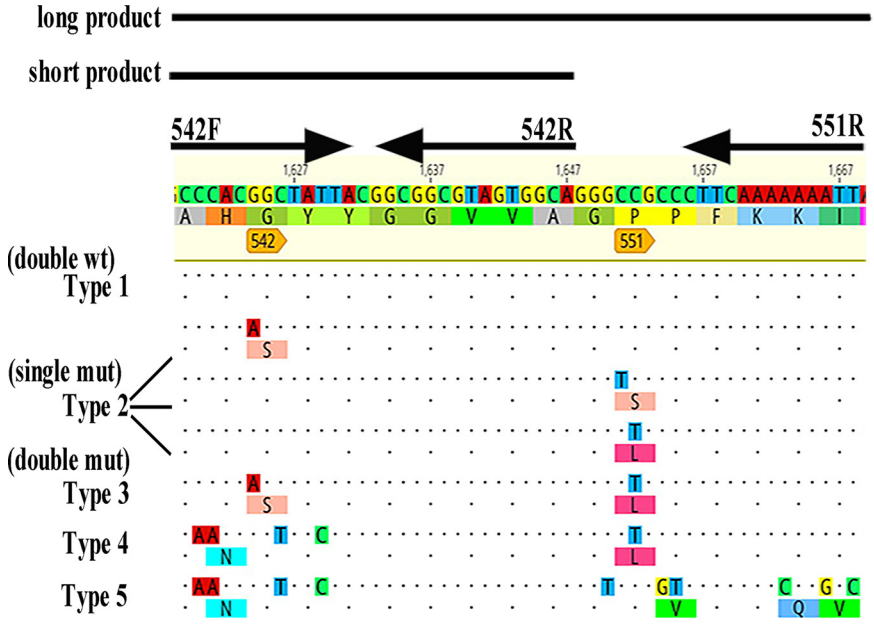
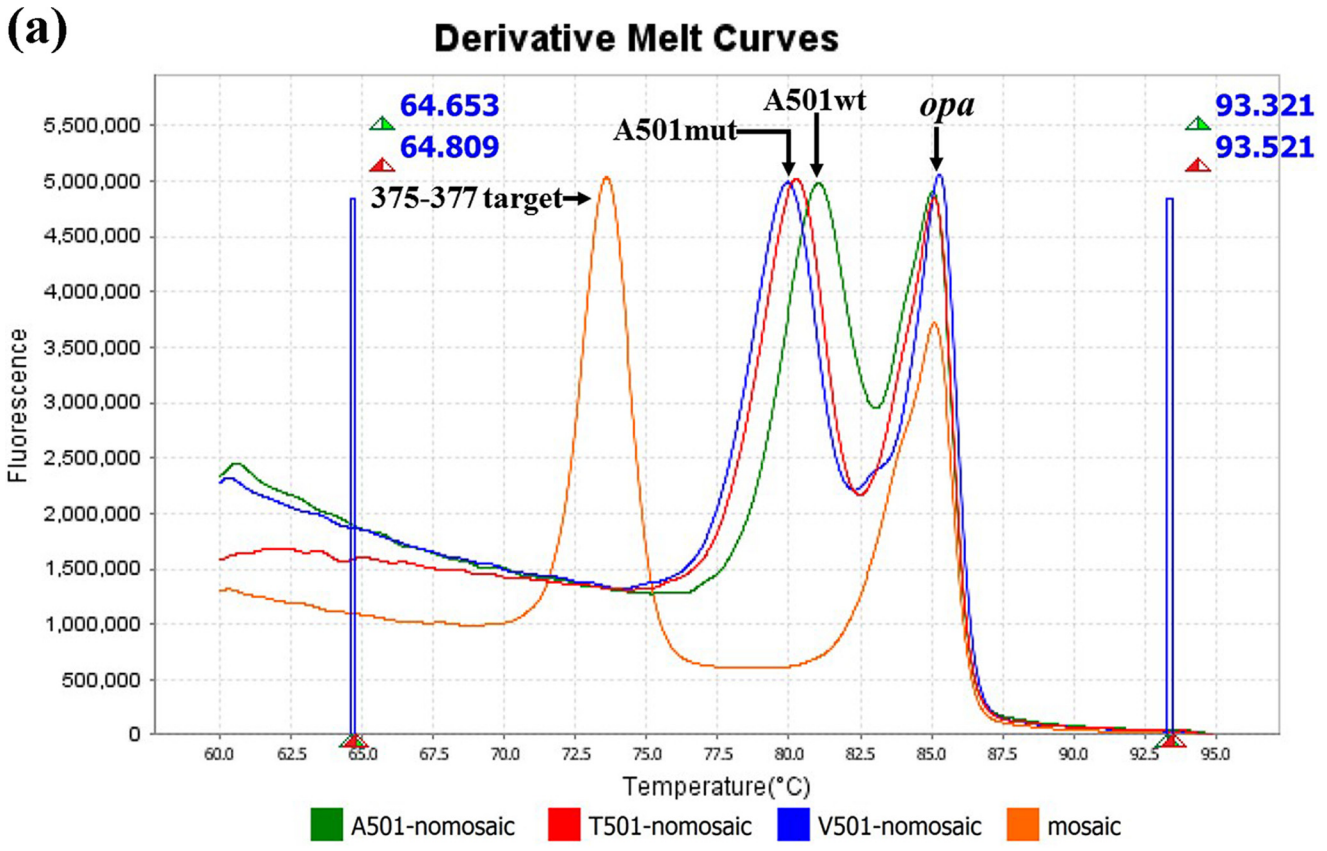


FIG 1 Illustration of the sequence structure from position 542 to 551 and the principle underlying primer design.



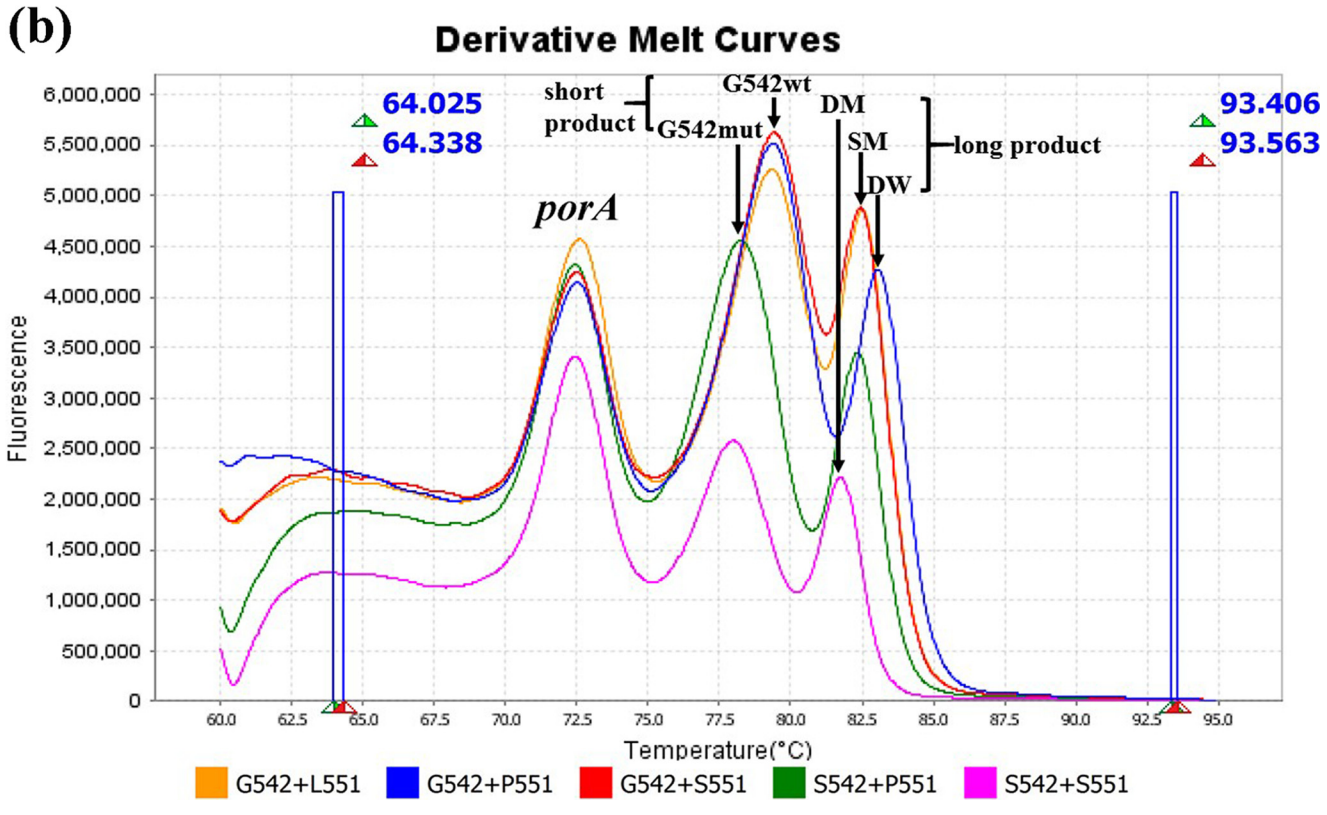


FIG 2 Results of the multiplex HRM assay. (a) HRM curve analysis of targets 375 through 377 and 501 for Assay 1. (b) HRM curve analysis of targets 542 and 551 for Assay 2. HRM, high-resolution melting; mut, mutation type; wt, wild type; DM, double mutations (PenA_542mut + PenA_ 551mut); SM, single mutation (PenA_542wt + PenA_551mut)/(PenA_542mut + PenA_551wt); DW, double wild-type (PenA_542wt + PenA_551wt).

TABLE 2 Tm values of target genes[a]

Purpose	Target	Position	Mutation	Tm	
				Range	Mean ± SD
Mosaic type					
	penA	375–377	Mosaic	73.71–73.78	73.74 ± 0.02
			Nonmosaic	NA	NA
Nonmosaic type	*penA*	501			
			Mosaic	NA	NA
			501wt(A)	80.95–81.01	80.99 ± 0.02
			501mut(T)	79.94–79.99	79.96 ± 0.02
			501mut(V)	79.48–79.56	79.51 ± 0.02
		542			
		short product	542wt(G)	79.45–79.52	79.47 ± 0.02
			542mut(S)	77.89–78.03	77.97 ± 0.04
		551			
		long product	Mosaic	NA	NA
			551mut (Type 4)	NA	NA
			double-wt		
			542wt + 551wt	83.11–83.22	83.17 ± 0.03
			Single-mut		
			542mut + 551wt	82.30–82.36	82.33 ± 0.03
			542wt + 551mut	82.48–82.57	82.52 ± 0.03
			double-mut		
			542mut + 551mut	81.72–81.87	81.82 ± 0.05
Species identification	*opa*	*N. gonorrhoeae*		85.57–85.63	85.60 ± 0.02
		Non-gonococcal *Neisseria spp.*		NA	NA
	porA	*N. gonorrhoeae*		72.45–72.59	72.53 ± 0.05
		Non-gonococcal *Neisseria spp.*		NA	NA

[a]wt, wild type; mut, mutated type.

(Table S4). The primary results (success rate: 98–100%) indicated that the present method was suitable for swabs and urine samples, suggesting its potential direct applicability to clinical samples without culture.

Molecular characterization of all isolates. As shown in Table S5, there was a significant difference in the proportion of decreased susceptibility to CRO (CRO-DS) between mosaic type (82.6%) and nonmosaic type (29.8%), indicating that mosaic *penA* is an effective assay target for screening CRO-DS isolates (χ^2 test, $P < 0.01$). Notably, 97.8% of CRO-DS isolates and 91.2% of CFM-DS isolates harboring A501mut and P551mut showed greater efficiency in developing CRO-DS (64.4%) (χ^2 test, $P < 0.01$) than CFM-DS (42.9%) (χ^2 test, $P > 0.05$).

The A501mut, which accounted for the largest proportion (73.5%), showed low power in independently improving the rate of CRO-DS and CEM-DS isolates. However, isolates with either P551mut or G542mut showed a significant increase in the proportion of CRO-DS and CEM-DS only in the presence of A501mut (② vs. ⑥ and ④ vs. ⑧) (Figure 3a). As shown in Figure 3b, the combination of A501mut and G542mut/P551mut increased the number of CRO-DS and CEM-DS isolates and increased the MIC of all samples by 1- to 2-fold for both antibiotics. Notably, among all isolates with typical resistance (MIC = 0.25 mg/L), 8/10 (80%) CRO-DS isolates contained A501mut and P551mut, whereas no apparent differences were

TABLE 3 Comparison of the performance of the 6-codon HRM assay with that of Sanger sequencing for identification of the AMR determinants of *Neisseria gonorrhoeae*[a]

No. of samples	AMR site	No. of samples with the following result:				Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
		TMT	FMT	TWT	FWT				
Total (n = 346)	375–377	14	0	332	0	100 (73.2–100)	100 (98.5–100)	100 (73.2–100)	100 (98.5–100)
Nonmosaic (n = 332)	501	109	0	223	0	100 (95.8–100)	100 (97.9–100)	100 (95.8–100)	100 (97.9–100)
	542	84	0	248	0	100 (94.6–100)	100 (98.1–100)	100 (94.6–100)	100 (98.1–100)
	551	107	0	224	1	99.1 (94.2–100)	100 (97.9–100)	100 (95.7–100)	99.6 (97.1–100)

[a]TMT, true mutation type; FMT, false mutation type; TWT, true wild type; FWT, false wild type; PPV, positive predictive value; CI, confidence interval; AMR, antimicrobial resistance; NPV, negative predictive value; HRM, high-resolution melting.

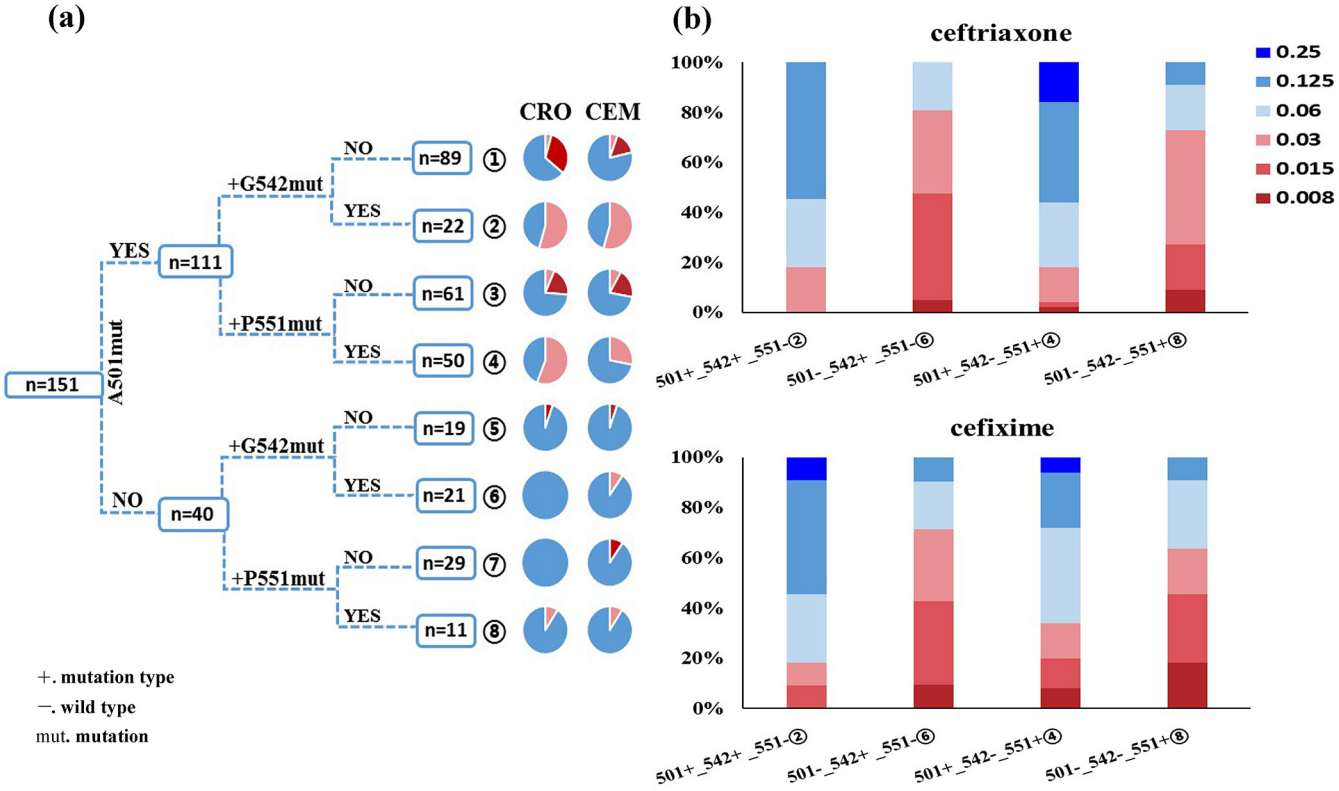


FIG 3 (a). The molecular characterization of 151 isolates with nonmosaic *penA*. Blue, susceptibility isolates; pink, isolates with decreased susceptibility; red, for ① ⑤, isolates with decreased susceptibility harboring P551mut, and for ③ ⑤, isolates with decreased susceptibility harboring G542mut. (b) MIC distribution of single and double mutations.

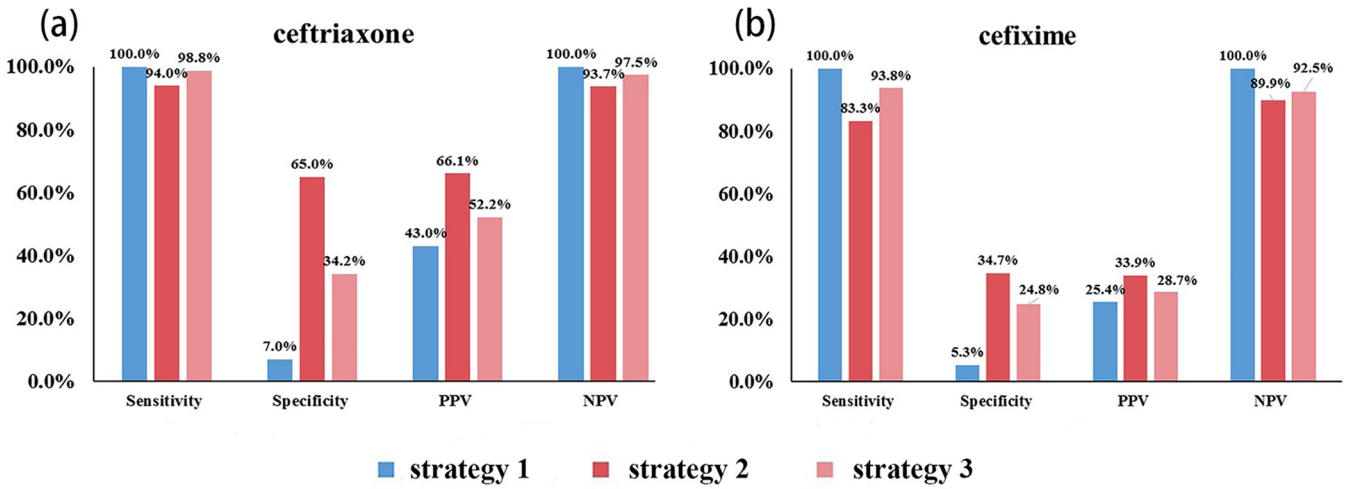
observed between the two groups (A501mut_G542mut versus A501mut_P551mut) among CEM-DS.

Prediction of CEP susceptibility using the 6-codon method. To evaluate the effectiveness of AMR prediction using the 6-codon method, we calculated the negative predictive value (NPV), positive predictive value (PPV), specificity, and sensitivity for 197 isolates using the MIC profiles of CEP (including cefixime and CRO). Based on genetic analysis of all isolates, we devised three detection strategies and analyses: strategy 1, detecting mosaic *penA* or at least one mutation among A501mut, G542mut, and P551mut in nonmosaic *penA*; strategy 2, detecting mosaic *penA* or A501mut plus either G542mut or P551mut; and strategy 3, detecting mosaic *penA* or A501mut in the nonmosaic *penA* gene.

The NPV, PPV, specificity, and sensitivity of each strategy are shown in Fig. 4. Strategy 1 showed 100% sensitivity (95% CI: 94.5–100%) and 100% NPV (95% CI: 59.8–100%) for both CRO and CEM and suboptimal specificities of $7\%_{CRO}$ (95% CI: 3.2–13.8%) and $5.3\%_{CEM}$ (95% CI: 2.5–10.7%), suggesting that the method produced a certain number of positive results with a susceptible phenotype. Surprisingly, there was a significant increase in specificity (CRO, 7% → 65%; CEM, 5.3% → 34.7%) when maintaining good sensitivity, indicating that this strategy can filter out a large number of false-positive results as compared with strategy 1. For strategy 3, both sensitivity and specificity were between the respective results for strategies 1 and 2 (Fig. 4). In summary, all three strategies showed high sensitivity, with strategy 2 showing the highest specificity.

DISCUSSION

Because a high monitoring capacity has never been reached, there is a shortage of critical AMR information. Moreover, because of the long turnaround time using culture methods for *N. gonorrhoeae*, current AMR-surveillance studies can only provide retrospective information, and the treatment guidelines based on this information always lag behind



Performance of 6-codon HRM assay for AMR prediction among three strategies.

Antimicrobial	Strategy	TP	FP	TN	FN	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Ceftriaxone	1	83	106	8	0	100 (94.5-100)	7.0 (3.2-13.8)	43.0 (36.8-51.3)	100 (59.8-100)
	2	78	40	74	5	94.0 (85.9-97.8)	65.0 (55.3-73.5)	66.1 (56.7-74.4)	93.7 (85.2-97.6)
	3	82	75	39	1	98.8 (62.5-99.9)	34.2 (25.7-43.8)	52.2 (44.1-60.2)	97.5 (85.3-99.9)
Cefixime	1	48	141	8	0	100 (90.8-100)	5.3 (2.5-10.7)	25.4 (19.5-32.3)	100 (59.8-100)
	2	40	78	71	8	83.3 (69.2-92.0)	34.7 (39.5-56.0)	33.9 (25.6-43.3)	89.9 (80.5-95.2)
	3	45	112	37	3	93.8 (81.8-98.4)	24.8 (18.3-32.7)	28.7 (21.9-36.5)	92.5 (78.5-98.0)

PPV, positive predictive value; NPV, negative predictive value.

FIG 4 Resistance prediction of the three different decision strategies.

current AMR status. Therefore, as a significant technical supplement to AMR monitoring in *N. gonorrhoeae*, the 6-codon method is valuable in providing comprehensive AMR epidemiological data for the timely revision of guidelines and tracking the emergence and transmission of resistance in many countries.

In the present study, the data showed that 29.8% of CRO-DS isolates and 22.5% of CEM-DS isolates harbored a nonmosaic *penA*. Such high proportions suggested considering nonmosaic *penA*-related resistance in our molecular assay to avoid losing any important resistant isolates. Furthermore, a previous systematic review demonstrated that 97.1% of nonmosaic *penA* isolates harbored A501mut, G542mut, and/or P551mut, which was also verified in the present study with 100% sensitivity (18). Consequently, several findings provide strong evidence for the three most efficient molecular markers among nonmosaic *penA* isolates (A501mut, G542mut, and P551mut), and we can further adjust the decision strategy based on local resistance data to optimize the performance of this method (5, 18). Compared with strategy 1, the results showed that strategy 2 significantly decreased the false-positive rate of detecting CRO-DS (93% $\rightarrow$ 35%) and CEM-DS (94.7% $\rightarrow$ 65.3%) (χ^2 test, $P < 0.001$) isolates, which greatly improved the efficiency of the primary screening.

With looming CEP-resistant isolates, many patients seek empirical antimicrobial treatment without a pretest for resistance, which is an urgent threat to antimicrobial stewardship (20). Hence, rapid and reasonable AMR-prediction methods are urgently required. Because the CEP-resistance mechanism of *N. gonorrhoeae* is multifactorial, the critical problem

with previous molecular assays is the detection of as many molecular markers as possible (such as MtrRmut, PorBmut, and PenA_G545mut) without an effective screening strategy for resistant isolates (15, 21, 22). Most of these molecular markers are insufficient to confer decreased susceptibility independently, indicating that we cannot efficiently screen isolates with reduced susceptibility at the molecular level, which is also the main reason why NAAT technology can only be used as a supplement to culture methods in AMR surveillance (18, 23). Previous modeling work has indicated that "abandoned" antimicrobial agents (cefixime) could be reused as long as the frequency of use and AMR emergence are strictly monitored, reinforcing the urgency to establish powerful real-time AMR-prediction methods (24). Therefore, in this study, we devised a multiplex HRM assay based on the 6-codon assay that showed high sensitivity and specificity and demonstrated accurate discrimination of distinct mutations in the *penA* allele while meeting the requirements of high-throughput and low-cost AMR monitoring under different settings. To the best of our knowledge, this is the first molecular assay with high generalizability, which is supported by a systematic review of data for all CEP-DS isolates from public databases. The results suggest that culture methods can be replaced with NAATs for AMR monitoring.

The main limitation of the HRM assay is the intrinsic restriction of the primers, as the type 4 nonmosaic *penA* (Fig. 1) (represented by *penA*-120.001 and *penA*-109.001) failed to provide valid data for assay 2. However, both *penA* types are only reported sporadically worldwide and are not associated with a resistance phenotype; therefore, we believe that this limitation is outweighed by the high sensitivity, low cost, and robust performance of our assay for predicting CEP-DS isolates. A further limitation is that the method described in this study only screens isolates with decreased susceptibility but not resistance. In a previous study, we designed a singleplex HRM assay to identify resistant clones by targeting the A311V alteration in the *penA*-60.001 gene, with this serving as a supplementary assay for the 6-codon method. If necessary, after performing the 6-codon method, all isolates with mosaic *penA* can be further tested using the singleplex HRM assay to detect the known fundamental alterations associated with CRO resistance (25).

In conclusion, we designed a multiplex molecular assay based on HRM analysis that provides an efficacious and cost-effective tool for predicting decreased susceptibility to CEPs. This method has the advantages of high sensitivity, specificity, and throughput, and its low cost makes it affordable in any region ($<$1.00 USD per sample). Additionally, this is the first assay based on a comprehensive sequence database review, which provides an ideal pretest tool for clinical antimicrobial stewardship and sets the stage for future molecular assay development.

MATERIALS AND METHODS

Isolates and antimicrobial susceptibility testing. To establish the HRM assay, 346 *N. gonorrhoeae* isolates were collected as control isolates from the National Center for STD Control, Chinese Center for Disease Control and Prevention. Furthermore, the *penA* gene profiles of all isolates were identified using the Sanger sequencing prior to HRM analysis in this study. Primers are listed in Table S1. Different genotypes were selected for each AMR site in the *penA* gene (375–377, 501, 542, 551), and each sample was evaluated repeatedly at least 12 times to obtain the Tm range.

In addition, 197 isolates with MIC profiles of cephalosporin (including cefixime and ceftriaxone) were selected to evaluate the performance of cephalosporin-resistant prediction, which were all provided by the Shenzhen Center for Chronic Disease Control. The MIC profiles were acquired using an agar dilution method as previously reported (26), and the cut-off value of decreased susceptibility to cephalosporin (cefixime and ceftriaxone) was MIC $\geq$ 0.125 mg/L (5, 18, 19, 27).

Optimization of the multiplex assay. For assay 1, three primer pairs were designed to flank specific single nucleotide polymorphisms at positions 375–377 and 501 in the *penA* allele, which is strongly related to cephalosporin resistance. Unfortunately, it was not possible to include two of the three positions (PenA_501, 542, and 551) in the same assay because they were too close to each other, causing nontarget amplification. Therefore, we downloaded all published *penA* sequences from the NG-STAR website (https://ngstar.canada.ca/, last accessed July 8, 2021) and performed an in-depth analysis of the *penA* structure. Fortunately, we found that the sequence from position 542 to 551 in *penA* of all 197 *penA* sequence types exhibited an apparent regularity, which could be summarized in terms of five structure types. These structure types are illustrated in Fig. 1. Therefore, in assay 2, we provided a forward primer (penA_542F) and two reverse primers (penA_542R and penA_551R) to produce a long product and a short product with high and low (Tm) values, respectively. Thus, we determined whether the sequence from 542 to 551 was wild type (PenA_542wt + PenA_551wt) or a mutant

form (including double mutation, PenA_542mut + PenA_551mut, or a single mutation, PenA_542wt + PenA_551mut or PenA_542mut + PenA_551wt) by calculating the Tm value of the long product.

Additionally, the Tm value of the short product can directly and precisely identify the PenA_G542S mutation. We can then differentiate diverse combinations of alleles at positions 542 and 551 using a "subtraction" between the two products. The principle of primer design is shown in Fig. 1. For species identification, the *opa* and *porA* genes were added to assays 1 and 2, respectively. The optimal primers and reaction conditions are listed in Table 1. For all amplicons, the theoretical Tm value was assessed in the design phase using the online tool OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html). The validity of primers targeting positions 375–377 was evaluated using nine important mosaic *penA* types, and another nine common nonmosaic *penA* types were selected to assess the primers at positions 501, 542, and 551. The details of all the types used are listed in Table S2.

Commensal *Neisseria* spp. strains and clinical samples. We used 11 nongonococcal *Neisseria* spp. isolates to assess the potential cross-reactivity of our method: *Neisseria polysaccharea* ($n = 5$), *Neisseria sicca* ($n = 1$, ATCC_29193), *Neisseria mucosa* ($n = 1$, ATCC_49233), *Neisseria flavescens* ($n = 1$, ATCC_13120), *Neisseria lactamica* ($n = 1$, ATCC_49142), and *Neisseria meningitidis* ($n = 2$). In total, 153 clinical samples (141 urethral swabs and 12 urine samples) confirmed as positive for *N. gonorrhoeae* by real-time PCR were collected to evaluate the performance of the 6-codon method (it should be noted that no culture data, and hence no MIC profiles for cefixime and CRO, were available for these clinical samples). All clinical samples were quantified by real-time PCR using the quantitative standard curve method and primers and probes listed in Table S3 (details concerning construction of the standard curve are provided in the Table S3 footnote).

Detection limit of the 6-codon method. The number of the genome copies of the two isolates (one mosaic type and one nonmosaic type) was assessed by real-time PCR using the quantitative standard curve method to assess the limit of detection of the 6-codon method (primers and probes are listed in Table S3, with details concerning construction of the standard curve provided in the footnote). Furthermore, the two isolates were serially diluted to concentrations of 5,000 copies/μL, 500 copies/μL, 250 copies/μL, 100 copies/μL, 50 copies/μL, 25 copies/μL, 5 copies/μL, and 1 copy/μL (10,000, 1000, 500, 200, 100, 50, 10, and 2 copies per reaction). Every isolate with a known quantity of genome copies was repeatedly tested 10 times to obtain a stable detection limit.

HRM analysis. Cycling parameters were as follows: denaturation for 10 min at 94°C, followed by 35 cycles at 95°C for 15 s, and 60°C for 1 min. For the HRM stage, the temperature was maintained at 60°C for 1 min and then increased by 0.025°C/s to 95°C for fluorescence measurements. The multiplex HRM assay was performed using the QuantStudio 6 and 7 Flex real-time PCR platform (Applied Biosciences, Foster City, CA, USA).

Statistical analysis. The Tm value and 95% confidence intervals (CIs) were calculated using SPSS software (v.17.0; SPSS Inc., Chicago, IL, USA) with 10 test replicates. The parameters of PPV, NPV, specificity, sensitivity, and the 95% CIs of each strategy were calculated for comparison with the culture method using the VassarStats website (http://vassarstats.net/index.html).

Ethics statement. The study protocol was approved by the Institutional Review Board of the Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China. In accordance with the Helsinki Declaration, all participants provided written informed consent.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We declare no conflicts of interest.

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