ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2009, p. 4211–4216 Vol. 53, No. 10
0066-4804/09/$08.00+0 doi:10.1128/AAC.00385-09

Simple, Rapid, and Inexpensive Detection of *Neisseria gonorrhoeae* Resistance Mechanisms Using Heat-Denatured Isolates and SYBR Green-Based Real-Time PCR▽

Gayle Kugelman,[1,2,3] John W. Tapsall,[4] Namraj Goire,[1,2,3] Melanie W. Syrmis,[1,2,3] Athena Limnios,[4] Stephen B. Lambert,[1,2,3] Michael D. Nissen,[1,2,3,5,6] Theo P. Sloots,[1,2,3,5,6] and David M. Whiley[1,2,3]*

Queensland Children's Medical Research Institute, Infectious Diseases Laboratory, Royal Children's Hospital, Herston Road, Herston, QLD 4029, Queensland Australia[1]; Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital and Health Service District, Herston, Queensland, Australia[2]; Clinical Medical Virology Centre, University of Queensland, Queensland, Australia[3]; WHO Collaborating Centre for STD and HIV, Microbiology Department, South Eastern Area Laboratory Services, Prince of Wales Hospital, Sydney, New South Wales, Australia[4]; Microbiology Division, Pathology Queensland, Royal Brisbane Hospital Campus, Brisbane, Queensland, Australia[5]; and Department of Paediatric and Child Health, University of Queensland, Brisbane, Queensland, Australia[6]

Received 22 March 2009/Returned for modification 28 April 2009/Accepted 6 June 2009

Neisseria gonorrhoeae **has developed resistance to multiple classes of antimicrobials. There is now growing concern that without the availability of appropriate public health strategies to combat this problem, gonorrhea could become untreatable. For this reason, surveillance for gonococcal antimicrobial resistance must be optimal both in terms of obtaining a representative sample of gonococcal isolates and in terms of having the appropriate tools to identify resistance. To aid with this surveillance, molecular tools are increasingly being used. In the present study, we investigated the use of a simple heat denaturation protocol for isolate DNA preparation combined with SYBR green-based real-time PCR for the identification of mutations associated with *N. gonorrhoeae* antimicrobial resistance. A total of 109 clinical gonococcal isolates were tested by high-resolution melting (HRM) curve analysis for chromosomal mutations associated with gonococcal resistance to beta-lactam antibiotics: a *penA* 345A insertion, *ponA* L421P, *mtrR* G45D, substitutions at positions 120 and 121 in *porB1b*, and an adenine deletion in the *mtrR* promoter. An allele-specific PCR assay was also investigated for its ability to detect the adenine deletion in the *mtrR* promoter. The results were compared to those obtained by DNA sequencing. Our HRM assays provided the accurate discrimination of heat-treated isolates in which the sequence types differed in GC content, including isolates with the *penA* 345A insertion and the *ponA* L421P and *mtrR* G45D mutations. The allele-specific PCR assay accurately identified isolates with the adenine deletion in the *mtrR* promoter. Heat-denatured DNA combined with SYBR green-based real-time PCR offers a simple, rapid, and inexpensive means of detecting gonococcal resistance mechanisms. These methods may have broader application in the detection of polymorphisms associated with phenotypes of interest.**

Neisseria gonorrhoeae is the etiologic agent of the sexually transmitted disease gonorrhea, causing an estimated 60 million new infections annually (7). In females, gonorrhea is a major cause of pelvic inflammatory disease and may lead to tubal infertility, ectopic pregnancy, and chronic pelvic pain, whereas in males it primarily causes urethritis (2, 3, 7). The control of gonorrhea is achieved through the provision of effective antimicrobial therapy to eradicate the infection, reduce transmission, and prevent the development of complications. However, *N. gonorrhoeae* has managed to develop resistance to multiple classes of antimicrobials, including penicillins and tetracyclines, and quinolones have been withdrawn from use in many parts of the world because of widespread resistance. Even the more recently available macrolides now have limited utility, leaving extended-spectrum cephalosporins (both oral and injectable) and spectinomycin (when it is available) as the mainstays of treatment. Without appropriate public health strategies to combat this problem, there is a real threat that gonorrhea will become untreatable (18). The Centers for Disease Control and Prevention has recently expressed fears regarding the ability to control gonorrhea, the need to explore alternate treatment options as a response to the increasing cephalosporin resistance in *N. gonorrhoeae*, and the requirement for enhanced monitoring of gonococcal resistance globally (4, 30).

The volatile nature of antimicrobial resistance in gonococci means that surveillance for resistance for public health purposes must be optimal both in terms of obtaining a sufficiently large and representative sample of gonococcal isolates and in terms of using the appropriate tools to identify resistance (19). The increasing use of nucleic acid amplification assays in industrialized countries and the widespread application of syndromic management principles in less developed countries have increasingly restricted the availability of gonococcal isolates for the phenotypic detection of resistance rates. Logistical

* Corresponding author. Mailing address: Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital & Health Service District, Herston Road, Herston, Queensland 4029, Australia. Phone: 61-7-3636 1623. Fax: 61-7-3636 1401. E-mail: d.whiley@uq.edu.au.

▽ Published ahead of print on 15 June 2009.

TABLE 1. Oligonucleotides used in this study

Assay and primer name	Sequence	Target	Position[a]	Product size (bp)
HRM curve analysis				
penA-345A-F	CGTCTCCCGTGCGCGA	*penA*	1516143–1516128	38 (wild type), 41 (345A insertion)
penA-345A-R	CAAAGAGGGGTAAACATGGGTA	*penA*	1516103–1516124	
ponA1-L421P-F	GTGGTTCAAGAGCCGTTG	*ponA*	109910–109927	38
ponA1-L421P-R	GAAACCAAAGCCCCCTG	*ponA*	109947–109931	
mtrR-G45D-F	CCGGCGTAACGCGC	*mtrR*	1327916–1327929	39
mtrR-G45D-R	TTTTGAAATGCCAATAGAGCGC	*mtrR*	1327954–1327933	
porB-120-F	CAGCCCCCTGAAAAACACC	*porB*	1789036–1789054	46
porB-121-R	GGATTCCCAAGCATTGACGTT	*porB*	1789081–1789061	
*mtrR*prom-F	TATACATACACGATTGCACGGA	*mtrR* promoter	1327718–1327739	49 (wild type), 48 (A deletion)
*mtrR*prom-R	AGGGCGGATTATAAAAAAGAC	*mtrR* promoter	1327766–1327746	
Allele-specific PCR				
*mtrR*prom-ASP-F1	CATACACGATTGCACGGATAAAA	*mtrR* promoter	1327722–1327744	65 (wild type), 64 (A deletion)
*mtrR*prom-ASP-F2	TACACGATTGCACGGATAAAAAG	*mtrR* promoter	1327724–1327746	63 (wild type), 62 (A deletion)
*mtrR*prom-ASP-R	CGTTTCGGGTCGGTTTGA	*mtrR* promoter	1327786–1327769	

[a] Positions in the complete genome of *N. gonorrhoeae* FA1090 (GenBank accession number AE004969).

problems with gonococcal storage and transport and the intrinsic fragility of *N. gonorrhoeae* also have adverse effects on the availability of viable isolate (21, 31).

Molecular tools are being used to provide an understanding of the genetic basis of resistance as well as to supplement phenotypic antimicrobial susceptibility testing (9, 15, 16, 20, 22, 23, 24). These tools have not yet advanced to a stage where they can be used in place of phenotypic testing, mainly because the genetic determinants of resistance to most antibiotics are not yet fully known. Nevertheless, for well-characterized resistance mechanisms, molecular tools offer an accurate and objective means of detection and systems for the detection of pivotal resistance determinants for public health purposes when viable isolates are not required have been described (22, 23).

An ongoing limitation of molecular assays is that they can be prohibitively expensive. The advent of real-time PCR technology has, for the most part, alleviated the need for costly DNA sequencing. However, real-time PCR assays targeting gonococcal resistance mechanisms have to date predominantly combined initial DNA extraction with probe-based real-time PCR. Both of these factors can impinge on the cost-effectiveness of the use of real-time PCR; commercial DNA extraction methods can be expensive, and the use of fluorophore-labeled probes can involve considerable initial setup costs. This is particularly the case when multiple mutations are being investigated, as different probe sets specific for each mutation need to be purchased. Furthermore, we have found that the accuracy of probe-based real-time PCR can be undermined by unexpected variations in the sequences of the probe targets (28, 29). This is particularly relevant to *N. gonorrhoeae*, given that the species comprises numerous subtypes that exhibit considerable sequence diversity (25, 27) as well as because some of the genes implicated in conferring resistance, including *porB1b*, are highly variable.

In the study described here, we sought to develop a simple and cost-effective means of identifying mutations associated with gonococcal resistance for potential applications in screening for antimicrobial resistance for public health purposes. We did this using two approaches: first, we sought to use a simple heat denaturation protocol rather than DNA extraction for isolate DNA preparation to decrease costs and the preparation time. This also allows the examination of nonviable organisms. Second, we used SYBR green-based real-time PCR, thereby removing the need for the initial purchase of expensive fluorophore-labeled probes and allowing the use of only a single primer pair. Briefly, the study was conducted by developing high-resolution melting (HRM) curve analysis assays targeting five key chromosomal mutations associated with gonococcal resistance to beta-lactam antibiotics, including an Asp-345A codon insertion in the *penA* gene, which encodes penicillin binding protein 2 (PBP 2) (5, 6); the L421P substitution in the *ponA* gene, which encodes PBP 1 (13); a G45D substitution in the encoding region of the *mtrR* gene (32); an adenine deletion in the *mtrR* promoter region (32); and various substitutions in amino acids 120 and 121 of the *porB1b* protein (8, 12). An allele-specific PCR assay was also investigated for its ability to detect the adenine deletion in the *mtrR* promoter.

MATERIALS AND METHODS

***N. gonorrhoeae* strains and heat denaturation.** A total of 109 gonococcal clinical isolates were selected and tested using the real-time PCR assays. These isolates were selected on the basis of their geographical and temporal diversity and ceftriaxone MIC and formed part of the isolates used in a previous study (26). Briefly, the isolates included local and international strains and comprised 13 isolates from various parts of Asia, with the remaining 96 strains coming from various regions of the Australian state of New South Wales. The years of isolation were 1988 ($n = 3$), 1997 ($n = 1$), 1999 ($n = 1$), 2000 ($n = 3$), 2001 ($n = 12$), 2002 ($n = 11$), 2003 ($n = 7$), 2004 ($n = 12$), and 2005 ($n = 59$). Heat denaturation was performed by selecting three to six colonies from a 24-h single-colony subculture of each isolate, suspending the colonies in 1.0 ml of distilled water in a 1.5-ml tube, and heating the tubes at 100°C for 8 min.

Primer design. For the HRM curve analysis-based real-time PCR assays, primers were designed to flank the sequences or the codons of interest. For PCR assays targeting the *penA* 345A insertion and the *ponA1* L421P and *mtrR* G45D substitutions, the forward and reverse primers directly flanked a single codon

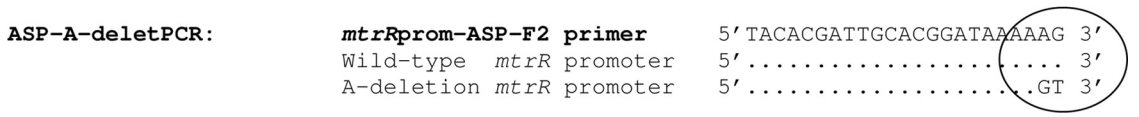

FIG. 1. Primer design for the *mtrR* promoter allele-specific PCR. The sequences of all primers except primer *mtrR*prom-ASP-F2 matched 100% both the wild-type sequence and the sequence with the adenine deletion; primer *mtrR*prom-ASP-F2 had two mismatches at the 3′ end (circled) compared to the sequence with the adenine deletion.

(primers *penA*-345A-F and *penA*-345A-R, *ponA1*-L421P-F and *ponA1*-L421P-R, and *mtrR*-G45D-F and *mtrR*-G45D-R, respectively; Table 1). For the detection of substitutions at positions 120 and 121 of the *porB1b* protein, a single primer pair (primers *porB*-120-F and *porB*-121-R) was used to directly flank both codons (Table 1). For the *mtrR* promoter assay, primers were designed to flank the region with the adenine deletion (primers *mtrR*prom-F and *mtrR*prom-R; Table 1). The PCR product sizes for these assays ranged from 38 to 49 bp (Table 1). The assays were designed so that different sequence types could be distinguished by HRM curve analysis.

An allele-specific primer (ASP)-based method was also designed to detect the adenine deletion in the *mtrR* promoter. This method used two real-time PCRs for each isolate. The first reaction (the ASP-based control PCR [ASP-control-PCR]) used forward primer *mtrR*prom-ASP-F1 and reverse primer *mtrRprom*-ASP-R (Table 1), which were designed to have a 100% match with the *mtrR* promoter sequences of both wild-type gonococcal strains and gonococcal strains with the adenine deletion (Fig. 1). The ASP-controlPCR therefore served as the amplification control. The second reaction (the ASP-based PCR for the detection of the adenine deletion [ASP-A-deletPCR]) used the same reverse primer as the ASP-controlPCR but used forward primer *mtrR*prom-ASP-F2. Compared to the sequence of primer *mtrR*prom-ASP-F1, the sequence of primer *mtrR*prom-ASP-F2 was moved up the target sequence by 2 bases, placing the primer target across the region with the adenine deletion. Primer *mtrR*prom-ASP-F2 therefore matches 100% with the sequence of wild-type gonococci, but the 2 bases on the extreme 3′ end mismatch the sequence of gonococci with the adenine deletion (Fig. 1). This mismatch resulted in an identifiable delay in the ASP-A-deletPCR cycle threshold (C_T) value compared to the ASP-controlPCR C_T value for isolates with the adenine deletion.

Reaction mixture and cycling conditions. All HRM assays and allele-specific real-time PCRs were performed with a standard reaction mixture and standard cycling conditions. Each reaction mixture contained 10.0 μl of 2× SYBR green quantitative PCR SuperMix-UDG (Invitrogen, Australia), 0.5 μM of forward and reverse primers, and 2.0 μl of heat-denatured isolate and was made up to a total volume of 20.0 μl with DNase-free water. Cycling was performed on a RotorGene 6000 instrument (Corbett Life Science, Australia) with the following two-step cycling conditions: an initial enzyme activation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s and primer annealing and elongation at 60°C for 30 s, with the fluorescence signal being read on green at 60°C following extension. HRM curve analysis was performed following PCR amplification; the reaction mixtures were continuously analyzed from 60°C to 95°C with temperature increments of 0.5°C/s.

Control specimens. Appropriate heat-denatured isolate controls of known sequence types, as determined by DNA sequencing, were included for all test runs. For the assay for the detection of substitutions at positions 120 and 121 in *porB1b*, eight isolate controls of different sequence types were used: GS (codons GGCAGC), DA (GACGCC), KG (AAGGGC), KD (AAGGAC), KN (AAGAAC), NA (AACGCC), and two different GA sequence types (GA1 and GA2; codons GGTGCC and GGCGCC, respectively).

For the remaining assays there were two sequence controls for each test run. These included the *penA* wild type and the *penA* 345A insertion (codon CGA), *ponA* L421 (CTG) and *ponA1* 421P (CCG), *mtrR* G45 (GGC) and *mtrR* 45D (GAC), and the *mtrR*promoter wild type and the *mtrR*promoter adenine deletion. Two negative (no-DNA) controls were also included in each test run.

Real-time PCR result interpretation. Analysis of the HRM assay results was performed with RotorGene 6000 HRM software (Corbett Life Science) with a confidence setting of 90%. In each run, the results for the test isolates were compared to those for the respective controls.

For the *mtrR* promoter allele-specific PCR, the results were determined on the basis of the C_T values. Briefly, the difference in C_T values observed in the ASP-A-deletPCR and ASP-controlPCR assays was determined for each isolate. This delta C_T value was then used to assign the sequence type.

DNA sequencing. The results of the HRM and allele-specific real-time PCR assays were compared to the results of DNA sequencing of the *penA*, *ponA*, *mtrR*, *porB*, and *mtrR* promoter sequences for all 109 isolates. Briefly, previously described primers were used to amplify the *ponA* (GAAAATGGGGGAGGACCGTA and TTAAAACAGGGAATCCAACTGC [13, 14]) and *mtrR* (AACAGGCATTCTTATTTCAG and TTAGAAGAATGCTTTGTGTC [11]) sequences by PCR. The PCR products were sent to the Australian Genome Research Facility (http://www.agrf.org.au/) for automated fluorescent sequencing. The *penA* and *porB* sequences for all isolates were available from a previous study (26).

RESULTS

HRM curve analysis. The results for the HRM real-time PCR assays compared to those of DNA sequencing for 109 isolates are summarized in Table 2. The results of HRM assay detection of the wild-type *ponA* and mutated *ponA1* sequences provided 100% agreement with the results of DNA sequencing for all 109 isolates. For *mtrR* codon 45, the HRM assay correctly identified 22 isolates with the G45D mutation and 86 isolates with the wild-type sequence. However, one isolate with a G45S sequence (codon AGC) was incorrectly assigned a G45D sequence type (GAC) by the HRM assay. For the *mtrR* promoter sequence, the results of the HRM assay and DNA sequencing were in agreement for 103 of the 109 isolates: 77 isolates with the A deletion and 26 isolates with the wild-type sequence. However, four isolates with the A deletion were incorrectly assigned to the wild type by the HRM method, and one isolate with the wild-type sequence was incorrectly assigned as having an A deletion. One isolate with an A deletion could not be assigned a sequence type by the *mtrR* promoter HRM assay.

The *penA* HRM assay correctly identified all 95 isolates displaying the 345A codon insertion; however, it failed to assign a sequence type for 11 of 14 wild-type isolates. These 11 isolates were not assigned a sequence type by the HRM analysis software because their sequences failed to be amplified by the *penA* assay. DNA sequencing showed that all 11 isolates possessed the previously described mosaic *penA* sequence (1, 26). The failure to be amplified was due to primer mismatches with the mosaic *penA* sequence (Table 3). For analysis of the *porB1b* codons at positions 120 and 121, HRM analysis correctly identified isolates with sequence types GA2, KD, KG, KN, and NA but could not readily distinguish sequence types DA, GA1, and GS. An additional five isolates were not assigned a sequence type by the *porB1b* HRM assay. These isolates possessed *porB1a* (and not *porB1b*) genes and could

TABLE 2. Summary of HRM real-time PCR and DNA sequencing results

Sequence	No. isolates (n = 109)	Sequence type by:		HRM confidence (%)		Melting temp (°C)		No. of GC bases (% GC content)
		DNA sequencing	HRM real-time PCR	Range	Mean, median	Range	Mean, median	
ponA	94	L421P (CCG)	L421P (CCG)	93.5–100	98.9, 99.6	79.25–79.85	79.58, 79.5	23 (60.5)
	15	Wild type (CTG)	Wild type (CTG)	98.1–100	99.8, 99.9	78.5–78.9	78.64, 78.65	22 (57.9)
penA	95	345A (CGA)	345A (CGA)	94.8–99.7	98.9, 99.4	77.75–78.1	77.95, 77.9	24 (58.5)
	3	Wild type	Wild type	99.1–99.6	99.5, 99.6	76.6	76.6, 76.6	22 (57.9)
	11[a]	Mosaic[a]	Variant[a,b]	No confidence data[a]	No confidence data[a]	No melting curve[a]	No melting curve[a]	
mtrR	22	G45D (GAC)	G45D (GAC)	98.4–100	99.6, 99.8	77.4–77.6	77.5, 77.5	22 (56.4)
	1	G45S (AGC)	G45D (GAC)	100		77.5		22 (56.4)
	86	Wild type (GGC)	Wild type (GGC)	94.8–100	99.5, 99.8	78.65–79	78.79, 78.75	23 (59.0)
mtrR promoter	77	A deletion	A deletion	96.5–100	99.1, 99.4	70.75–70.9	70.76, 70.75	17 (35.4)
	1	A deletion	Variant	No confidence data	No confidence data	70.5		17 (35.4)
	4	A deletion	Wild type	94.1–98.5	97.2, 98.1	70.5–70.75	70.65, 70.75	17 (35.4)
	26	Wild type	Wild type	94.1–100	98.7, 98.9	70.5–70.75	70.62, 70.6	17 (34.7)
	1	Wild type	A deletion	97.3		70.75		17 (34.7)
porB	3	DA (GACGCC)	DA (GACGCC)	98.9–99.7	99.3, 99.2	78.12–78.37	78.27, 78.3	26 (56.5)
(amino acids 120 and 121)	4	DA (GACGCC)	GA1 (GGTGCC)	91.4–99.9	97.7, 99.6	78.1–78.3	78.23, 78.22	26 (56.5)
	5	GA1 (GGTGCC)	GA1 (GGTGCC)	99–99.9	99.5, 99.4	78.1–78.25	78.2, 78.22	26 (56.5)
	4	GA2 (GGCGCC)	GA2 (GGCGCC)	95.2–99.8	98.4, 98.9	79.18–79.38	79.28, 79.25	27 (58.7)
	2	GS (GGCAGC)	DA (GACGCC)	99.2–99.6	99.4, 99.2	78.35–78.37	78.36, 78.35	26 (56.5)
	4	GS (GGCAGC)	GS (GGCAGC)	98.5–99.8	99.2, 99.2	78.32–78.53	78.38, 78.33	26 (56.5)
	75	KD (AAGGAC)	KD (AAGGAC)	90.2–99.9	97.3, 97.7	75.77–76.42	76.18, 76.2	24 (52.2)
	1	KG (AAGGGC)	KG (AAGGGC)	99.0		77.15		25 (54.3)
	2	KN (AAGAAC)	KN (AAGAAC)	99.1–99.3	99.2, 99.1	75.3–75.4	75.35, 75.3	23 (50.0)
	4	NA (AACGCC)	NA (AACGCC)	98.1–99.1	98.4, 98.1	77.45–77.52	77.49, 77.5	25 (54.3)
	5	*porB1a*	Variant[c]	No confidence data[c]	No confidence data[c]	No melting curve[c]	No melting curve[c]	

[a] These isolates contained the mosaic *penA* sequence, and their sequences were not amplified in the *penA* HRM assay due to mismatches in the primer targets.
[b] Variant, could not be assigned a sequence type by the HRM assay.
[c] These isolates contained a *porB1a* gene, and their sequences were not amplified in the *porB1b* HRM assay.

not be amplified in the assay because of primer mismatches (data not shown). The results of DNA sequencing also showed that numerous other sequence mismatches were present in the primer targets of the HRM assays but that these mismatches did not have an effect on the sequence type results assigned by HRM curve analysis (Table 3).

Allele-specific PCR. By the allele-specific PCR, the 27 isolates with the wild-type *mtrR* promoter sequence were readily distinguished from the 82 isolates with the A deletion on the basis of the delta C_T values from the ASP-controlPCR and the ASP-A-deletPCR; isolates with the wild-type sequence (as determined by DNA sequencing) provided delta C_T values rang-

TABLE 3. Examples of sequence variations in primer targets and impact upon results of real-time PCR assays

No. of isolates	Assay	Primer target(s)	Sequence type	Sequence	Impact on PCR assay results
11	*penA* 345-A HRM assay	Both primers	Forward primer	5′-CGTCTCCCGTGCGCGA-3′	Variant[a] (failed to amplify)
			Isolate *penA*	5′-..G..A....A.AA..-3′	
			Reverse primer	5′-CAAAGAGGGGTAAACATGGGTA-3′	
			Isolate *penA*	5′-.....TA..A.....G.....-3′	
3	*mtrR* G45D HRM assay	Forward primer	Primer	5′-CCGGCGTAACGCGC-3′	None
			Isolate *mtrR*	5′-.............A.-3′	
9	*mtrR* G45D HRM assay	Reverse primer	Primer	5′-TTTTGAAATGCCAATAGAGCGC-3′	None
			Isolate *mtrR*	5′-................T.....-3′	
4	*porB* HRM assay	Reverse primer	Primer	5′-GGATTCCCAAGCATTGACGTT-3′	None
			Isolate *porB*	5′-..................T..-3′	
5	*porB* HRM assay	Reverse primer	Primer	5′-GGATTCCCAAGCATTGACGTT-3′	None
			Isolate *porB*	5′-.............CC.....-3′	
1	*mtrR* promoter ASP assay	Forward primer	Primer	5′-CATACACGATTGCACGGATAAAA-3′	None
			Isolate *mtrR* promoter	5′-.............G..........-3′	
1	*mtrR* promoter ASP assay	Forward primer	Primer	5′-CATACACGATTGCACGGATAAAA-3′	None
			Isolate *mtrR* promoter	5′-....A................-3′	

[a] Variant, could not be assigned a sequence type by the HRM assay.

ing from 0.1 to 2.5 cycles (mean, 0.6 cycles; median, 0.4 cycles), whereas isolates with the A deletion provided delta C_T values ranging from 5.1 to 12.9 cycles (mean, 9.7 cycles; median, 9.8 cycles). Sequence variation was observed in the forward primer for two isolates with the wild-type promoter sequence, but this did not have an effect on the results of the allele-specific PCR (Table 3).

DISCUSSION

Our results show that the *penA* 345A, *ponA* L421P, and *mtrR* G45D HRM assays and the *mtrR* promoter allele-specific PCR were accurate in distinguishing the sequence types of heat-treated isolates. The results of the *penA* 345A and *ponA* L421P HRM assays and the *mtrR* promoter allele-specific PCR were in complete agreement with the results of DNA sequencing. Only one incorrect *mtrR* G45D HRM curve analysis result was observed.

These results show the HRM assays provide the accurate discrimination of heat-treated isolates, so long as the sequence types differ in their GC contents. The one isolate incorrectly assigned to the G45D type by the *mtrR* HRM assay possessed a rare AGC (G45S) codon (23) and therefore had the same GC content and base composition as the G45D (GAC) sequence type. Likewise, the *mtrR* promoter HRM assay could not readily distinguish the *mtrR* promoter sequence types, which differed by only a single adenine deletion, and the *porB* HRM assay had difficulty distinguishing the eight different *porB1b* sequence types, some of which overlapped in GC contents. To investigate the *porB* HRM analysis issue further, we reanalyzed the *porB* HRM curve analysis data by excluding the data for isolates and controls with genotypes GS, KG, and GA1, such that the remaining five sequence types had different GC contents: KN (AAGAAC), KD (AAGGAC), NA (AACG CC), DA (GACGCC), and GA2 (GGCGCC). Upon reanalysis, all five genotypes were accurately distinguished by the HRM assay (data not shown). This confirms that the HRM methods can readily distinguish multiple sequence types when heat-denatured isolates are used, so long as the GC contents of the sequence types are different. To further investigate the *mtrR* promoter HRM assay, we performed several repeat test runs and found that the incorrect sequence type assignment of the assay was not restricted to particular isolates but in fact occurred more randomly (data not shown). This further highlights the limited ability of the approach to accurately distinguish sequence types of similar GC content. Previous studies have shown that the HRM assay is capable of distinguishing different sequence types of the same GC content when purified DNA is used at a defined concentration (10, 17). There is no technical reason why the HRM assay could not be used to accurately distinguish the eight sequence types associated with the *porB1b* codons at positions 120 and 121 or the single adenine deletion in the *mtrR* promoter investigated in this study. However, the aim of this study was to find a simple and cost-effective means of identifying mutations associated with gonococcal resistance. The use of heat-denatured isolates rather than purified DNA for this purpose reduced the discriminatory power of HRM curve analysis. Despite this limitation, we found that the HRM assay could successfully be used with heat-denatured isolates for the detection of a range of key mutations. Furthermore, we found that allele-specific PCR was a suitable alternative when the HRM assay was unsuccessful, given that it accurately identified the sequence types of the *mtrR* promoter sequence for all isolates. This shows that the combined used of heat-denatured isolates with the HRM assay and allele-specific PCR offers a simple, inexpensive, and rapid means of detecting a broad range of mutations: each SYBR green reaction costs approximately Aus$1.50 and could be performed in less than 2 h, including the time for isolate heat treatment. It should be noted that the use of a DNA extraction method would otherwise have increased the cost by a minimum of Aus$4.00 per isolate and would have added approximately 30 min to the sample preparation time.

A notable advantage of detection by the HRM assay observed in this study was that the assay design was relatively simple, as it has limited primer design requirements. The primers need to directly flank only the mutations of interest and need to have a sufficient melting temperature to operate at an annealing temperature of 60°C under two-step cycling conditions. In fact, we found that strong primer dimers, which were evident in the G45D HRM assay primers, had no observable effect on the results of typing by the HRM assay. This was presumably because the gonococcal DNA load was sufficiently high for the PCR to favor the specific reaction, limiting the potential for nonspecific reactions to occur.

A further benefit of the HRM approach was that the sequence type results assigned by the HRM assays were largely unaffected by sequence variation in the regions flanking the targeted mutations. Mismatches were observed in the HRM assay primer targets for several isolates, yet this did not result in the assignment of an incorrect sequence type by the HRM method. This can be attributed to the fact that upon amplification the primer sequences are incorporated into the resulting PCR product, and therefore, a PCR product can differ in its sequence composition only if the sequences internal to the primer targets are different. This is in contrast to probe-based methods, for which we have previously shown that unexpected sequence variations can have a significant impact on hybridization probe-based assays, given that these methods require a relatively large region of conserved sequence (generally about 50 bases) to accommodate both probes (28). It is highly unlikely that a hybridization probe-based approach would have been suitable for many of the targeted mutations in this study, given the extent of the sequence variation observed. The only obvious impact that sequence variation in primer targets did have on the HRM assays was for isolates with the previously described mosaic *penA* sequence and those with the *porB1a* genes, which failed to be amplified in the *penA* 345A and *porB* HRM assays, respectively. We do not consider this to be a major limitation, given that these isolates were identified as variants by the HRM methods, enabling them to be flagged for DNA sequencing or testing by an alternative detection method. It should be noted that unexpected mutations in primer targets do have the potential to affect the accuracy of allele-specific PCR results. However, such problems were not observed in this study, presumably because the two mutations that were observed in the forward primer of the *mtrR* promoter allele-specific PCR (Table 3) were not in the extreme 3′ end of the primer.

In conclusion, we found that the heat denaturation DNA

preparation method combined with SYBR green-based real-time PCR offers a simple, rapid, and inexpensive means of detecting *N. gonorrhoeae* mutations associated with antimicrobial resistance. The use of this approach has the potential to enhance public health-based surveillance for antimicrobial resistance in gonococci and to further our currently incomplete understanding of gonococcal genetic resistance mechanisms and the relationships between the different molecular changes and their effects on phenotypic resistance. Furthermore, we believe that these methods could be applied in other settings for the detection of polymorphisms associated with phenotypes of interest in microbiology and other areas of genetic research.

REFERENCES

1. **Ameyama, S., S. Onodera, M. Takahata, S. Minami, N. Maki, K. Endo, H. Goto, H. Suzuki, and Y. Oishi.** 2002. Mosaic-like structure of penicillin-binding protein 2 gene (*penA*) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. Antimicrob. Agents Chemother. **46:** 3744–3749.
2. **Biro, F. M., S. L. Rosenthal, and M. Kiniyalocts.** 1995. Gonococcal and chlamydial genitourinary infections in symptomatic and asymptomatic adolescent women. Clin. Pediatr. (Philadelphia) **34:**419–423.
3. **Centers for Disease Control and Prevention.** 2003. Sexually transmitted disease surveillance 2003. US Department of Health and Human Services, Atlanta, GA.
4. **Deguchi, T., and M. Yasuda.** 2008. Lack of nationwide surveillance of antimicrobial resistance in *Neisseria gonorrhoeae* in Japan. Ann. Intern. Med. **149:**363–364.
5. **Dougherty, T. J.** 1986. Genetic analysis and penicillin-binding protein alterations in *Neisseria gonorrhoeae* with chromosomally mediated resistance. Antimicrob. Agents Chemother. **30:**649–652.
6. **Dowson, C. G., A. E. Jephcott, K. R. Gough, and B. G. Spratt.** 1989. Penicillin-binding protein 2 genes of non-beta-lactamase-producing, penicillin-resistant strains of *Neisseria gonorrhoeae*. Mol. Microbiol. **3:**35–41.
7. **Gerbase, A. C., J. T. Rowley, D. H. L. Heymann, S. F. B. Berkley, and P. Piot.** 1998. Global prevalence and incidence estimates of selected curable STDs. Sex. Transm. Infect. **74:**S12–S16.
8. **Gill, M. J., S. Simjee, K. Al-Hattawi, B. D. Robertson, C. S. Easmon, and C. A. Ison.** 1998. Gonococcal resistance to ß-lactams and tetracycline involves mutation in loop 3 of the porin encoded at the *penB* locus. Antimicrob. Agents Chemother. **42:**2799–2803.
9. **Li, Z., S. Yokoi, Y. Kawamura, S. Maeda, T. Ezaki, and T. Deguchi.** 2002. Rapid detection of quinolone resistance-associated *gyrA* mutations in Neisseria gonorrhoeae with a LightCycler. J. Infect. Chemother. **8:**145–150.
10. **Liew, M., R. Pryor, R. Palais, C. Meadows, M. Erali, E. Lyon, and C. Wittwer.** 2004. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin. Chem. **50:**1156–1164.
11. **Mavroidi, A., L. S. Tzouvelekis, K. P. Kyriakis, H. Avgerinou, M. Daniilidou, and E. Tzelepi.** 2001. Multidrug-resistant strains of *Neisseria gonorrhoeae* in Greece. Antimicrob. Agents Chemother. **45:**2651–2654.
12. **Olesky, M., M. Hobbs, and R. A. Nicholas.** 2002. Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **46:**2811–2820.
13. **Ropp, P. A., M. Hu, M. Olesky, and R. A. Nicholas.** 2002. Mutations in *ponA*, the gene encoding penicillin-binding protein 1, and a novel locus, *penC*, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **46:**769–777.
14. **Shigemura, K., T. Shirakawa, N. Massi, K. Tanaka, S. Arakawa, A. Gotoh, and M. Fujisawa.** 2005. Presence of a mutation in *ponA1* of Neisseria gonorrhoeae in numerous clinical samples resistant to various beta-lactams and other, structurally unrelated, antimicrobials. J. Infect. Chemother. **11:** 226–230.
15. **Shigemura, K., T. Shirakawa, K. Tanaka, S. Arakawa, A. Gotoh, and M. Fujisawa.** 2006. Rapid detection of the fluoroquinolone resistance-associated ParC mutation in Neisseria gonorrhoeae using TaqMan probes. Int. J. Urol. **13:**277–281.
16. **Siedner, M. J., M. Pandori, L. Castro, P. Barry, W. L. Whittington, S. Liska, and J. D. Klausner.** 2007. Real-time PCR assay for detection of quinolone-resistant *Neisseria gonorrhoeae* in urine samples. J. Clin. Microbiol. **45:**1250–1254.
17. **Slinger, R., D. Bellfoy, M. Desjardins, and F. Chan.** 2007. High-resolution melting assay for the detection of *gyrA* mutations causing quinolone resistance in Salmonella enterica serovars Typhi and Paratyphi. Diagn. Microbiol. Infect. Dis. **57:**455–458.
18. **Tapsall, J.** 2006. Antibiotic resistance in Neisseria gonorrhoeae is diminishing available treatment options for gonorrhea: some possible remedies. Expert. Rev. Anti-Infect. Ther. **4:**619–628.
19. **Tapsall, J. W.** 2005. Antibiotic resistance in Neisseria gonorrhoeae. Clin. Infect. Dis. **41:**S263–S268.
20. **Unemo, M., P. Olcén, H. Fredlund, and S. Thulin.** 2008. Real-time PCR and subsequent pyrosequencing for screening of *penA* mosaic alleles and prediction of reduced susceptibility to expanded-spectrum cephalosporins in Neisseria gonorrhoeae. APMIS **116:**1004–1008.
21. **Unemo, M., O. Fasth, H. Fredlund, A. Limnios, and J. Tapsall.** 2009. 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. **63:**1142–1151.
22. **Vernel-Pauillac, F., V. Falcot, D. Whiley, and F. Merien.** 2006. Rapid detection of a chromosomally mediated penicillin resistance-associated *ponA* mutation in Neisseria gonorrhoeae using a real-time PCR assay. FEMS Microbiol. Lett. **255:**66–74.
23. **Vernel-Pauillac, F., S. Nandi, R. A. Nicholas, and C. Goarant.** 2008. Genotyping as a tool for antibiotic resistance surveillance of *Neisseria gonorrhoeae* in New Caledonia: evidence of a novel genotype associated with reduced penicillin susceptibility. Antimicrob. Agents Chemother. **52:**3293–3300.
24. **Whiley, D., J. Bates, A. Limnios, M. D. Nissen, J. Tapsall, and T. P. Sloots.** 2007. Use of a novel screening PCR indicates presence of Neisseria gonorrhoeae isolates with a mosaic *penA* gene sequence in Australia. Pathology **39:**445–446.
25. **Whiley, D. M., S. M. Garland, G. Harnett, G. Lum, D. W. Smith, S. N. Tabrizi, T. P. Sloots, and J. W. Tapsall.** 2008. Exploring 'best practice' for nucleic acid detection of Neisseria gonorrhoeae. Sex. Health **5:**17–23.
26. **Whiley, D. M., E. A. Limnios, S. Ray, T. P. Sloots, and J. W. Tapsall.** 2007. Diversity of *penA* alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob. Agents Chemother. **51:**3111–3116.
27. **Whiley, D. M., J. W. Tapsall, and T. P. Sloots.** 2006. Nucleic acid amplification testing for Neisseria gonorrhoeae: an ongoing challenge. J. Mol. Diagn. **8:**3–15.
28. **Whiley, D. M., and T. P. Sloots.** 2005. Melting curve analysis using hybridisation probes: limitations in microbial molecular diagnostics. Pathology **37:** 254–256.
29. **Whiley, D. M., and T. P. Sloots.** 2006. Sequence variation can affect the performance of minor groove binder TaqMan probes in viral diagnostic assays. J. Clin. Virol. **35:**81–83.
30. **Workowski, K. A., S. M. Berman, and J. M. Douglas, Jr.** 2008. Emerging antimicrobial resistance in Neisseria gonorrhoeae: urgent need to strengthen prevention strategies. Ann. Intern. Med. **148:**606–613.
31. **World Health Organization Gonococcal Antimicrobial Surveillance Programme.** 2008. Rationale and applications for the current (2008) WHO panel of Neisseria gonorrhoeae for antimicrobial resistance surveillance for public health purposes, and instructions for their use. Technical document D007-0408-1#1. WHO Collaborating Centre for STD, Sydney, Australia.
32. **Zarantonelli, L., G. Borthagaray, E. H. Lee, and W. M. Shafer.** 1999. Decreased azithromycin susceptibility of *Neisseria gonorrhoeae* due to *mtrR* mutations. Antimicrob. Agents Chemother. **43:**2468–2472.