

Rapid Detection of the Mosaic Structure of the *Neisseria gonorrhoeae penA* Gene, Which Is Associated with Decreased Susceptibilities to Oral Cephalosporins[∇]

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In *Neisseria gonorrhoeae*, the mosaic structure of the *penA* gene (encoding penicillin-binding protein 2 [PBP 2]), which is composed of fragments of the *penA* genes from *Neisseria cinerea* and *Neisseria perflava*, has been significantly associated with decreased susceptibility to cephalosporins, particularly oral cephalosporins. The aim of this study was to develop a rapid assay for the detection of mosaic PBP 2 of *N. gonorrhoeae* by real-time PCR. This assay successfully detected the mosaic *penA* gene of *N. gonorrhoeae*, and its sensitivity was $\geq 10^1$ copies/reaction. Six hundred twenty-one clinical strains were examined by this assay for the presence of mosaic PBP 2, which was detected in 85 (39.4%) of 216 strains from 2002, 69 (40.6%) of 170 strains from 2003, 71 (44.4%) of 160 strains from 2004, and 31 (41.3%) of 75 strains from 2005. The MICs of cephalosporins for strains with the mosaic PBP 2 detected by the assay were statistically higher than those for strains without the mosaic PBP 2. One hundred sixty-six (64.8%) of 256 strains with the mosaic PBP 2 exhibited cefixime MICs of ≥ 0.5 $\mu\text{g/ml}$. The emergence and spread of strains with mosaic PBP 2 could be a threat to the cefixime treatment of gonorrhea. This real-time PCR assay for the detection of mosaic PBP 2 of *N. gonorrhoeae* is thus useful in the prediction of decreased susceptibilities to oral cephalosporins.

Since the emergence and spread of fluoroquinolone-resistant *Neisseria gonorrhoeae*, cephalosporins have been used as the primary treatment for gonococcal infections. In Japan, however, clinical isolates of *N. gonorrhoeae* have been found to be less susceptible to oral cephalosporins, including cefixime (1, 7, 11). A mosaic penicillin-binding protein 2 (PBP 2) in clinical strains of *N. gonorrhoeae*, composed of fragments of PBP 2 from *Neisseria cinerea* and *Neisseria perflava*, has been reported to be associated with decreased susceptibility to oral cephalosporins (8). We have suggested that the decreased affinity of mosaic-structure recombinant PBP 2 for oral cephalosporins might contribute to the decreased susceptibility of *N. gonorrhoeae* to these antibiotics (12). In addition, recent studies have suggested that three amino acid substitutions in the mosaic PBP 2 might reduce the susceptibility of *N. gonorrhoeae* to cefixime (14) and that the *penA* mosaic structure, in conjunction with genetic polymorphisms in *mtrR*, *porB1b*, and *ponA*, might reduce the susceptibility to cefixime and ceftriaxone (9). The emergence and spread of strains with such a mosaic PBP 2 could threaten the possibility of treating gonorrhea with cephalosporins. Therefore, the detection of the *N. gonorrhoeae penA* gene encoding the mosaic PBP 2 associated with decreased susceptibilities to oral cephalosporins could be useful for identifying cephalosporin resistance in clinical strains of *N. gonorrhoeae*. However, to the best of our knowl-

edge, no studies of the detection of the mosaic *penA* gene have yet been reported. In the present study, we present our newly developed real-time PCR assay for the mosaic *penA* gene-specific amplification and detection of *N. gonorrhoeae* associated with decreased susceptibilities to cephalosporins.

MATERIALS AND METHODS

Bacteria. To develop a real-time PCR assay, we examined the type strains of *N. gonorrhoeae* (ATCC 19424), *N. cinerea* (ATCC 14685), and *N. perflava* (ATCC 10555) and four clinical strains of *N. gonorrhoeae* (GU01-12, GU01-29, GU01-89, and GU01-185) isolated from men with gonorrhea who were treated at Gifu University Hospital or its affiliated hospitals in central Japan in 2001. The clinical strains were included in our previous study, in which susceptibilities to penicillin, cephalosporins, and PBP 2 isoforms were analyzed (8). Compared to PBP 2 from ATCC 19424, GU01-12 and GU01-29 PBP 2 had the Asp-345a insertion (2). PBP 2 from GU01-89 and GU01-185 possessed a mosaic structure composed of fragments of PBP 2 from *N. cinerea* and *N. perflava*. To detect the mosaic PBP 2 in clinical strains, we examined 70 previously described clinical strains (8) and 621 clinical strains of *N. gonorrhoeae* isolated from men treated for gonorrhea at Gifu University Hospital or its affiliated hospitals in central Japan between 2002 and 2005. The MICs of cefozopran (Takeda, Osaka, Japan), cefdinir (Astellas Pharma, Inc., Tokyo, Japan), cefixime (Astellas Pharma), and ceftriaxone (Roche Diagnostics, Tokyo, Japan) for each strain were tested using the Clinical and Laboratory Standards Institute performance standards for antimicrobial susceptibility testing (3).

DNA extraction. For the *N. gonorrhoeae*, *N. cinerea*, and *N. perflava* strains and the four clinical strains of *N. gonorrhoeae*, DNAs were extracted by the phenol-chloroform method (10). The 70 previously described clinical strains and the 621 clinical strains of *N. gonorrhoeae* were dissolved in TE buffer (10 mM Tris, 1 mM EDTA; pH 8) and boiled for 10 min in a water bath, after which they were used directly as template DNA, without further purification of the DNA.

Preparation of standard DNA for real-time PCR. The 1,760-bp fragment including the *penA* gene was amplified with a pair of primers, *penA*-F2 (5'-GA ATTCTTGATTA AAAAGCGAATATAAGCCC-3') and *penA*-R3 (5'-GCGGC CGCTTAAGACGGTGT TTTGACGGCT-3'), as previously reported (12). The amplified 1,760-bp fragment of the mosaic structure of the GU01-89 *penA* gene was inserted into pAChLT-A (BD Biosciences, San Jose, CA) to generate *penA*/

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      . . . 810 . . . 820 . . . 830 . . . 840 . . . 850
NG (ATCC19424) TTTGGATGCCCGCACGGGGGAAATCCTCGCCTTGGCCAATACGCCCGCCT
NG (Mosaic)   G.....T..T.....A...T.....T...
NC (ATCC14685) G.....T..T.....T.....T.....T...
NP (ATCC10555) A.....G.AA..C..C.....TT.G..T...T...C.GC..T..T.

      . . . 860 . . . 870 . . . 880 . . . 890 . . . 900
NG (ATCC19424) ACGATCCCAACAGACCCGGCCGGGACAGACGGAACAGCGGGCAACCGT
NG (Mosaic)   .T..G.....A.....T.A.....A...T....C
NC (ATCC14685) .T..G.....A.....T.A.....A...T....
NP (ATCC10555) .....G..TCA...G..T.AA..CA.T.....C..T....

      . . . 910 . . . 920 . . . 930 . . . 940 . . . 950
NG (ATCC19424) GCCGTAACCGATATGATCGAACCTGGTTCGGCAATCAAACCGTTCGTGAT
NG (Mosaic)   .....C.....C.....T..C..G..G....TACC..
NC (ATCC14685) .....T....C.....G..C.....G..A..G..C..T..C..
NP (ATCC10555) .....T....C.....T....G.....TACC..

      . . . 960 . . . 970 . . . 980 . . . 990 . . .1000
NG (ATCC19424) TGCGAAGGCATTGGATGCGGGCAAAACCGATTGAAACGAACGGCTGAATA
NG (Mosaic)   ...C..A.....T.C.....GTG...GCA.C...TACAT.C....
NC (ATCC14685) .....A...C.....AT.....A.....GA..T.....
NP (ATCC10555) ...C..A.....T.C..T...GTG...GCA.C...TAC.T.C....

      . . .1010 . . .1020
NG (ATCC19424) CGCAGCCTTATAAAATCGGA
NG (Mosaic)   .C.T.....C.....T
NC (ATCC14685) .....T..T
NP (ATCC10555) .C.T.....C.....T

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FIG. 1. Locations of the primers and probe in the *penA* gene and the sequence of the *penA* genes from bases 801 to 1020. The light-gray arrows indicate the positions of the primers; the dark-gray shading indicates the position of the probe.

pAcHLT-A in TOP10 (Invitrogen, San Diego, CA) as previously reported (12). Next, *penA*/pAcHLT-A was purified with a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). The *penA*/pAcHLT-A was stored at -80°C ; its copy number was calculated by measuring the optical density at 260 nm.

Detection of the mosaic structure of the *N. gonorrhoeae penA* gene. Specific amplification of the mosaic structure of the *penA* gene was performed by TaqMan PCR with the primers NG89-F2 (5'-GTTGGATGCCCGTACTGGG-3'), which was the same as a sequence region in *N. cinerea*, and NG89-R1 (5'-ACC GATTTTGTAAAGGCAGGG-3'), which was the same as a sequence region in *N. perflava*. These were designed to specifically amplify the regions between nucleotides 801 and 1020 of the mosaic *penA* gene in *N. gonorrhoeae* and yielded a 220-bp fragment (Fig. 1). The probe NG89-P1 (5'-6-carboxyfluorescein-CGGC AAAGTGGATGCAACCGA-3'-6-carboxytetramethylrhodamine) was designed to detect mutations between nucleotides 968 and 989 of the mosaic *penA* gene (Fig. 1). The primers and probe were determined to be specific for the mosaic structure of the *N. gonorrhoeae penA* gene except in the *Neisseria meningitidis penA* gene (accession no. AY 294556) by online BLAST analysis on the National Center for Biotechnology Information website. The components of PCR in a final volume of 50 μl included TaqMan 1000 Reaction PCR core reagents (Applied Biosystems Japan, Tokyo, Japan) containing AmpliTaq Gold, AmpErase uracil *N*-glycosylase, 10 \times TaqMan Buffer A, deoxynucleoside triphosphate mix, and 25 mM MgCl_2 . The AmpliTaq Gold and AmpErase uracil *N*-glycosylase were added to final concentrations of 1.25 and 0.5 U/reaction, respectively. The primers, probe, and MgCl_2 were added to final concentrations of 1 μM , 250 nM, and 5 mM, respectively. Finally, 5 μl of template DNA was added to each reaction mixture. An ABI PRISM 7900 HT sequence detection system (Applied Biosystems Japan) was used for amplification and detection (2 min at 50°C , 15 min at 95°C , and 40 cycles of 15 s at 95°C and 60 s at 60°C).

Sequencing of the *penA* gene. The nucleotide sequence of the full-length *penA* genes from the *N. gonorrhoeae* clinical strains was determined as reported previously (8). The *penA* genes were sequenced by the dye terminator method

and with an automatic sequencer (model 3100; Applied Biosystems, Inc., Foster City, CA).

Statistical analysis. Mann-Whitney U tests were used to compare the MIC distributions. *P* values of less than 0.05 were considered significant.

RESULTS

Susceptibility to cephalosporins. The cephalosporin susceptibilities of the 621 strains analyzed in the present study are summarized in Table 1. There were no significant changes in the MIC distributions of cephalosporins for the clinical strains over 4 years.

Sensitivity. The 10-fold serially diluted plasmid DNA, *penA*/pAcHLT-A, was amplified by real-time PCR, and the 220-bp fragment was identified by ethidium bromide staining when the sample contained more than 1×10^1 copies/reaction. When the threshold cycles were plotted against the \log_{10} of the copy number of the *penA*/pAcHLT-A, linearity was observed over the range from 1×10^1 to 1×10^7 copies/reaction (Fig. 2). In one out of three reactions, 1×10^1 copies were detectable.

Specificity. The specificity of the assay to detect the mosaic *penA* gene was determined by testing DNAs from *N. gonorrhoeae* (ATCC 19424), *N. cinerea* (ATCC 14685), and *N. perflava* (ATCC 10555), as well as from the two clinical strains (GU01-12 and GU01-29) with the nonmosaic *penA* gene and the two clinical strains (GU01-89 and GU01-185) with the

TABLE 1. Cephalosporin susceptibilities of clinical strains of *N. gonorrhoeae* isolated in central Japan from 2002 to 2005

Cephalosporin and yr in which strains were isolated (no. of strains)	MIC ($\mu\text{g/ml}$)		
	Range	MIC ₅₀	MIC ₉₀
Cefozopran			
2002 (216)	0.015–32	0.5	16
2003 (170)	0.015–64	0.5	16
2004 (160)	0.008–32	0.5	16
2005 (75)	0.03–32	1	16
Cefdinir			
2002 (216)	0.004–2	0.06	1
2003 (170)	0.004–4	0.125	2
2004 (160)	0.004–2	0.06	1
2005 (75)	0.008–2	0.125	1
Cefixime			
2002 (216)	0.001–2	0.06	0.5
2003 (170)	0.002–2	0.06	1
2004 (160)	0.004–1	0.06	0.5
2005 (75)	0.004–1	0.125	0.5
Ceftriaxone			
2002 (216)	0.001–1	0.06	0.125
2003 (170)	<0.001–0.5	0.06	0.25
2004 (160)	<0.001–0.25	0.06	0.125
2005 (75)	0.002–2	0.03	0.125

mosaic *penA* gene. Signals in real-time PCR were detected only in GU01-89 and GU01-185.

Detection of the mosaic structure of the *N. gonorrhoeae penA* gene in clinical strains. Real-time PCR amplification was carried out on DNA isolated from the 70 previously described clinical strains and the 621 clinical strains isolated from 2002 to 2005. Real-time PCR identified the presence of the mosaic *penA* allele in all 47 of the 70 previously described clinical isolates that were shown by sequencing to contain the mosaic gene and did not detect the mosaic *penA* gene in the remaining 23 strains that were known not to contain the mosaic allele. The mosaic *penA* gene was detected in 256 strains, including 85 (39.4%) of 216 strains isolated in 2002, 69 (40.6%) of 170 strains isolated in 2003, 71 (44.4%) of 160 strains isolated in 2004, and 31 (41.3%) of 75 strains isolated in 2005.

Association of mosaic PBP 2 with MICs of cephalosporins. The strains with the mosaic PBP 2 detected by the present assay had statistically higher MICs than the nonmosaic strains for cefozopran, cefdinir, cefixime, and ceftriaxone from 2002 to 2005 (Fig. 3), with *P* values of less than 0.01. Two hundred forty-eight (96.9%) and 245 (95.7%) of the 256 strains with the mosaic PBP 2 exhibited cefozopran MICs in the range of 4 to 64 $\mu\text{g/ml}$ and cefdinir MICs in the range of 0.5 to 2 $\mu\text{g/ml}$, respectively, over the 4 years. Two hundred thirty-three (91.0%) of the 256 strains with the mosaic PBP 2 exhibited cefixime MICs in the range of 0.25 to 2 $\mu\text{g/ml}$. One hundred sixty-six (64.8%) exhibited cefixime MICs of 0.5 $\mu\text{g/ml}$ or greater than 0.5 $\mu\text{g/ml}$. The distributions of ceftriaxone MICs for the strains with the mosaic PBP 2 and those without the mosaic PBP 2 overlapped. However, the ceftriaxone MICs for strains with the mosaic PBP 2, ranging from 0.004 to 2 $\mu\text{g/ml}$, were significantly greater than those for strains without the mosaic PBP 2, which ranged from <0.001 to 0.5 $\mu\text{g/ml}$.

Sequencing of the *penA* gene. There were nine strains of *N. gonorrhoeae* detected by the real-time PCR assay that showed susceptibility to cephalosporins (cefzopran, MICs of <0.5 $\mu\text{g/ml}$; cefdinir, cefixime, or ceftriaxone, MICs of <0.25 $\mu\text{g/ml}$). Of these nine clinical strains, eight strains showed the mosaic structure and one strain showed a nonmosaic structure (Table 2). Compared to PBP 2 from GU01-12, this strain had an alteration of Asn-542→His. On the other hand, there were 12 strains of *N. gonorrhoeae* without the mosaic PBP 2 that showed decreased susceptibility to cephalosporins (cefzopran, MICs of ≥ 1 $\mu\text{g/ml}$; cefdinir, cefixime, or ceftriaxone, MICs of ≥ 0.5 $\mu\text{g/ml}$). In these clinical strains, the *penA* gene did not show the mosaic structure (Table 2).

DISCUSSION

We developed a real-time PCR assay for the detection of the mosaic structure of the *N. gonorrhoeae penA* gene, associated with decreased susceptibilities to cephalosporins. The real-time PCR assay targeting the mosaic structure of the *N. gonorrhoeae penA* gene was both highly specific and highly sensitive and could detect the mosaic PBP 2 in 256 of 621 clinical strains. A large number of clinical strains of *N. gonorrhoeae* could be examined for the presence of the mosaic PBP 2 without labor-intensive and time-consuming procedures, including sequencing of DNAs.

In our previous study, we sequenced the *penA* gene in 70 clinical strains of *N. gonorrhoeae*, finding this type of mosaic PBP 2 in 47 strains (8). In this previous study, all of the strains with the mosaic PBP 2 exhibited cefdinir MICs in the range of 0.25 to 2 $\mu\text{g/ml}$, while the remaining 23 strains, all possessing PBP 2 with Asp-345a or Asp-345a and additional amino acid alterations of one to six substitutions, showed cefdinir MICs in the range of 0.015 to 0.125 $\mu\text{g/ml}$. With respect to cefixime, 45 of 47 strains with the mosaic PBP 2 showed MICs in the range of 0.25 to 1 $\mu\text{g/ml}$ and two showed MICs of 0.125 $\mu\text{g/ml}$. The remaining 23 strains with nonmosaic PBP 2 showed cefixime MICs in the range of 0.015 to 0.125 $\mu\text{g/ml}$. The clinical strains were distinctly categorized into oral-cephalosporin-resistant and -susceptible types based on the presence or absence of mosaic PBP 2. In the present study, we examined 621 clinical strains for the presence of the mosaic PBP 2 by means of our developed assay. The MICs of cephalosporins for 256 strains with the mosaic PBP 2 detected by the assay were statistically higher than those for strains without the mosaic PBP 2. This assay could thus be a relevant tool for detecting the mosaic PBP 2 associated with decreased susceptibility to cephalosporins.

In this study, 64.8% of clinical strains with mosaic PBP 2 exhibited cefixime MICs of 0.5 $\mu\text{g/ml}$ or greater. We also reported that the emergence and spread of *N. gonorrhoeae* with the mosaic PBP 2 showing decreased susceptibility to cefixime could be a threat to the cefixime treatment for gonorrhea. In particular, therefore, this assay, which is able to detect the mosaic PBP 2 in *N. gonorrhoeae*, could be useful in screening for resistance to cefixime in clinical strains of *N. gonorrhoeae*.

With respect to ceftriaxone, in both our previous and present studies, the ceftriaxone MICs for strains with mosaic PBP 2 were significantly greater than those for strains with nonmosaic PBP 2. However, the distribution of the former

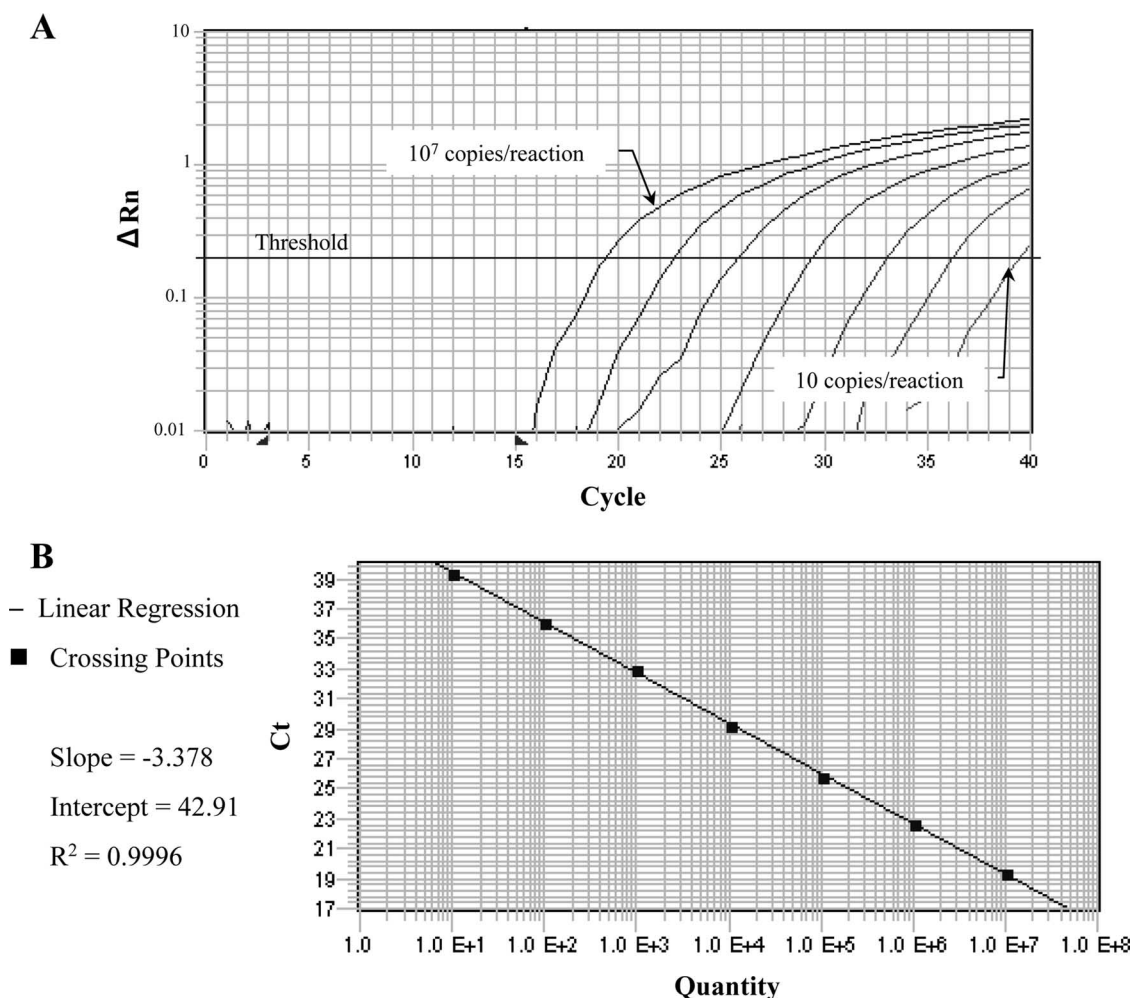


FIG. 2. (A) Fluorescence profiles of 10-fold serially diluted *penA*/pAChLT-A DNA from 1×10^1 to 1×10^7 copies/reaction analyzed by real-time PCR using an NG89-P1 probe. ΔR_n was calculated by subtracting the baseline fluorescence from the reporter fluorescence, which was normalized by an internal reference. (B) A standard curve plot of the 10-fold serial dilution of *penA*/pAChLT-A. Linearity is observed throughout the range from 1×10^1 to 1×10^7 copies/reaction.

MICs overlapped that of the latter MICs, and some strains with mosaic PBP 2 showed lower ceftriaxone MICs than did those with nonmosaic PBP 2. Recent studies suggest that the ceftriaxone-resistant type is not based on the presence of mosaic PBP 2 (15, 16). We also measured the affinity of mosaic-structure and non-mosaic-structure recombinant PBP 2 for cefdinir, cefixime, and ceftriaxone and found that the decreased affinity of mosaic-structure recombinant PBP 2 for oral cephalosporins might contribute to decreased susceptibility to these antibiotics in *N. gonorrhoeae* (12). Furthermore, we showed that mosaic-structure recombinant PBP 2 showed the same affinity for ceftriaxone as non-mosaic-structure recombinant PBP 2, suggesting that the susceptibility of ceftriaxone may be determined not only by alterations in PBP 2 but also by other mechanisms. Therefore, the detection of the mosaic structure of the *N. gonorrhoeae penA* gene in clinical strains could not be effective enough to predict resistance to ceftriaxone.

The present real-time PCR assay was significantly useful for detecting the mosaic PBP 2 in clinical strains of *N. gonor-*

rhoeae, which is indicative of decreased susceptibility to oral cephalosporins such as cefixime. However, this assay has several limitations. There were 12 strains of *N. gonorrhoeae* without the mosaic PBP 2 that showed decreased susceptibility to cephalosporins (cefazopran, MICs of $\geq 1 \mu\text{g/ml}$; cefdinir, cefixime, or ceftriaxone, MICs of $\geq 0.5 \mu\text{g/ml}$). In these clinical strains, the *penA* gene did not show the mosaic structure (Table 2), and susceptibility to cephalosporins appeared to be determined by other mechanisms (4–6, 13). On the other hand, nine strains of *N. gonorrhoeae* were detected by the real-time PCR assay that showed susceptibility to cephalosporins (cefazopran, MICs of $< 0.5 \mu\text{g/ml}$; cefdinir, cefixime, or ceftriaxone, MICs of $< 0.25 \mu\text{g/ml}$). All but one of these clinical strains had the mosaic-structure *penA* gene (Table 2). In these eight strains with the mosaic structure, the mosaic PBP 2 could not decrease susceptibility to cephalosporins. These nine strains, which include one strain for which the sequence pattern result was different from the real-time PCR, require further investigation. The decreased susceptibility to cephalosporins in these strains could not be screened by the assay. Another limitation

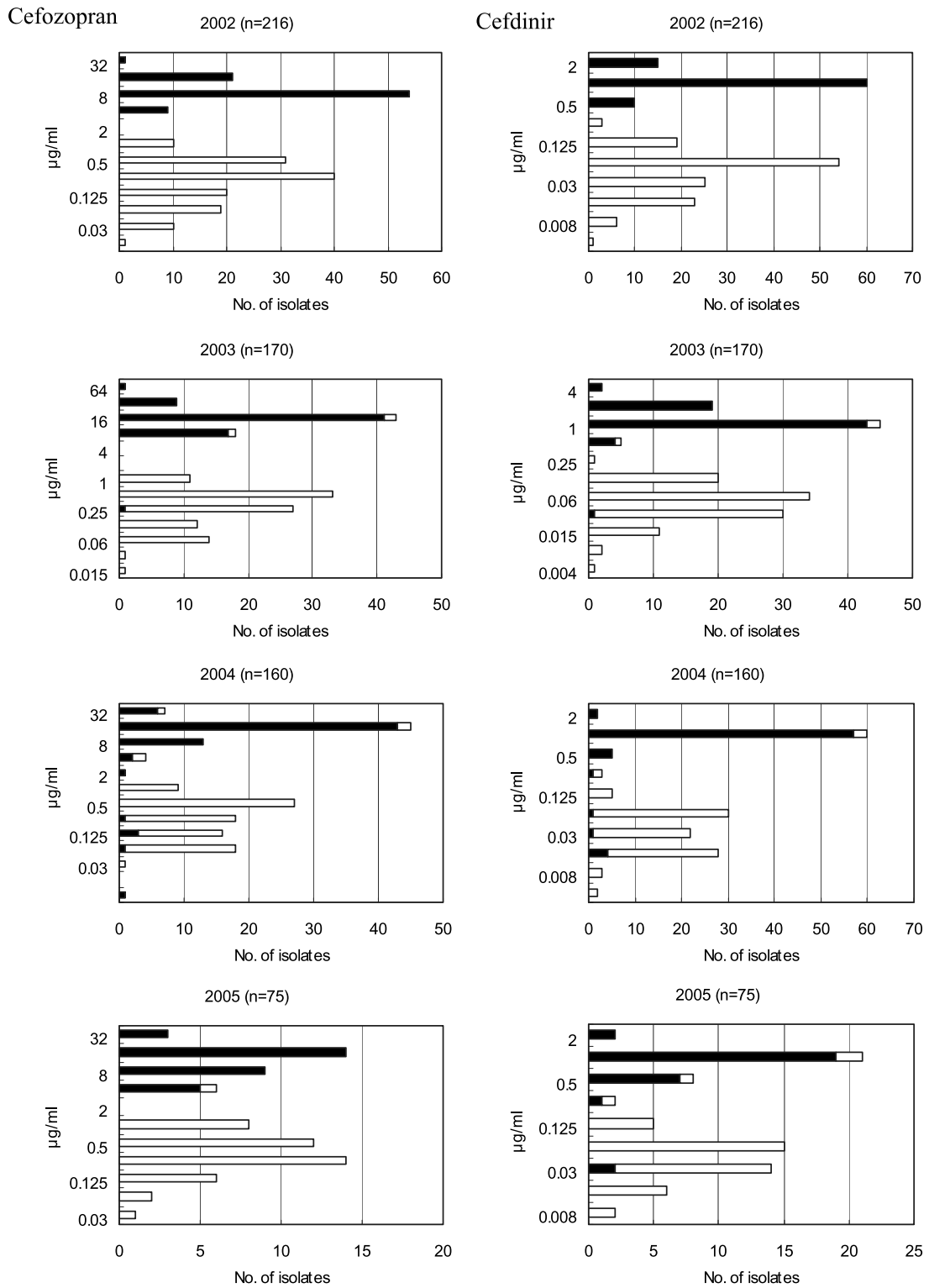


FIG. 3. Distributions of the MICs of ceftazidime, cefdinir, cefixime, and ceftriaxone for the clinical strains of *N. gonorrhoeae* from 2002 to 2005. The black bars indicate the number of strains with the mosaic PBP 2; the white bars indicate the number of strains without the mosaic PBP 2. Mann-Whitney U tests were used to compare the MICs for the strains with the mosaic PBP 2 with those for the strains without the mosaic PBP 2. The MICs of each cephalosporin for the strains with the mosaic PBP 2 were statistically higher than those for the strains without the mosaic PBP 2.

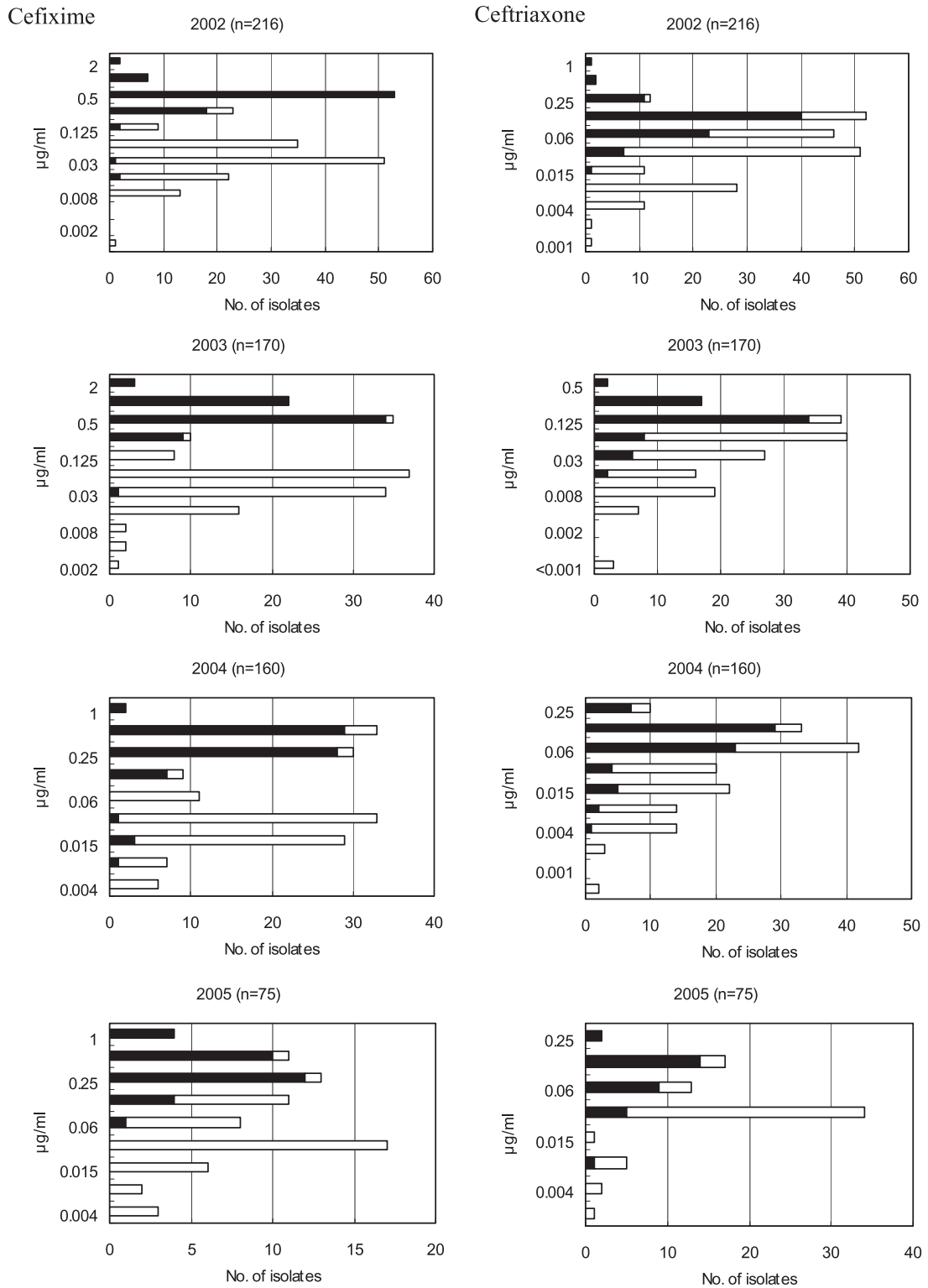


FIG. 3—Continued.

of the present assay was that the assay could not distinguish the mosaic *penA* gene of *N. gonorrhoeae* from the *N. meningitidis penA* gene (accession no. AY 294556), because the homology of AY 294556 to our primers (NG89-F2 and NG89-R1) and

probe (NG89-P1) was 100% in all cases. When the assay is used to detect mosaic PBP 2 of *N. gonorrhoeae* from clinical specimens, the discrimination between *N. gonorrhoeae* and *N. meningitidis* must be carried out by other tests. This real-time

TABLE 2. Sequence patterns of the strains for which the MIC and the real-time PCR results were in disagreement

Strain	Real-time PCR result (C_T) ^a	Sequence pattern	MIC (μ g/ml) of drug:			
			Cefozopran	Cefdinir	Cefixime	Ceftriaxone
GU03-252	+	Nonmosaic	0.25	0.03	0.03	0.015
GU04-001	+	Mosaic	0.125	0.015	0.015	0.008
GU04-061	+	Mosaic	0.125	0.015	0.015	0.015
GU04-063	+	Mosaic	0.06	0.015	0.008	0.004
GU04-068	+	Mosaic	0.25	0.06	0.03	0.06
GU04-091	+	Mosaic	0.125	0.015	0.015	0.008
GU04-207	+	Mosaic	0.008	0.03	0.125	0.015
GU05-012	+	Mosaic	16	0.03	0.25	0.06
GU05-016	+	Mosaic	4	0.03	0.25	0.03
GU03-065	-	Nonmosaic	8	1	0.5	0.125
GU03-201	-	Nonmosaic	16	1	0.06	0.06
GU03-204	-	Nonmosaic	16	0.5	0.03	0.06
GU04-052	-	Nonmosaic	16	1	0.5	0.125
GU04-060	-	Nonmosaic	32	1	0.5	0.25
GU04-062	-	Nonmosaic	16	1	0.5	0.125
GU04-244	-	Nonmosaic	4	0.25	0.5	0.25
GU04-312	-	Nonmosaic	4	0.25	0.25	0.25
GU05-011	-	Nonmosaic	0.25	1	0.06	0.03
GU05-013	-	Nonmosaic	0.25	1	0.03	0.03
GU05-018	-	Nonmosaic	0.25	0.5	0.5	0.03
GU05-062	-	Nonmosaic	4	0.25	0.25	0.125

^a C_T , threshold cycle; +, positive; -, negative.

PCR assay is a specific amplification and detection of the mosaic *penA* gene of *N. gonorrhoeae*; therefore, alterations in areas other than the amplification and detection region are undetectable. Nevertheless, the mosaic PBP 2 in clinical strains of *N. gonorrhoeae*, composed of fragments of PBP 2 from *N. cinerea* and *N. perflava*, was relevant to the decreased susceptibility to oral cephalosporins; the present real-time PCR assay was effective in predicting resistance to oral cephalosporins such as cefixime in clinical strains of *N. gonorrhoeae*. The amplification and detection region in this assay may contribute to the activity of transpeptidase. This assay is therefore useful as a rapid and simple screening tool in the surveillance of *N. gonorrhoeae* with decreased susceptibility to cephalosporins, particularly cefixime.

REFERENCES

1. Akasaka, S., T. Muratani, Y. Yamada, H. Inatomi, K. Takahashi, and T. Matsumoto. 2001. Emergence of cephem- and aztreonam-high-resistant *Neisseria gonorrhoeae* that does not produce beta-lactamase. *J. Infect. Chemother.* **7**:49-50.
2. Brannigan, J. A., I. A. Tirodimos, Q. Y. Zhang, C. G. Dowson, and B. G. Spratt. 1990. Insertion of an extra amino acid is the main cause of the low affinity of penicillin-binding protein 2 in penicillin-resistant strains of *Neisseria gonorrhoeae*. *Mol. Microbiol.* **4**:913-919.
3. Clinical and Laboratory Standards Institute. 2006. Performance standards for antimicrobial susceptibility testing; 16th informational supplement. M100-S16. Clinical and Laboratory Standards Institute, Wayne, PA.
4. Faruki, H., and P. F. Sparling. 1986. Genetics of resistance in a non-beta-lactamase-producing gonococcus with relatively high-level penicillin resistance. *Antimicrob. Agents Chemother.* **30**:856-860.
5. Gill, M. J., S. Simjee, K. Al-Hattawi, B. D. Robertson, C. S. Easmon, and C. A. Ison. 1998. Gonococcal resistance to beta-lactams and tetracycline involves mutation in loop 3 of the porin encoded at the *penB* locus. *Antimicrob. Agents Chemother.* **42**:2799-2803.
6. Hagman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd, and W. M. Shafer. 1995. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mirRCDE* efflux system. *Microbiology* **141**: 611-622.
7. Ito, M., M. Yasuda, S. Yokoi, S. Ito, Y. Takahashi, S. Ishihara, S. Maeda, and T. Deguchi. 2004. Remarkable increase in central Japan in 2001-2002 of *Neisseria gonorrhoeae* isolates with decreased susceptibility to penicillin, tetracycline, oral cephalosporins, and fluoroquinolones. *Antimicrob. Agents Chemother.* **48**:3185-3187.
8. Ito, M., T. Deguchi, K. S. Mizutani, M. Yasuda, S. Yokoi, S. Ito, Y. Takahashi, S. Ishihara, Y. Kawamura, and T. Ezaki. 2005. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. *Antimicrob. Agents Chemother.* **49**:137-143.
9. Lindberg, R., H. Fredlund, R. Nicholas, and M. Unemo. 2007. *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA*, *mirR*, *porB1b*, and *ponA*. *Antimicrob. Agents Chemother.* **51**:2117-2122.
10. Maniatis, T., J. Sambrook, and E. F. Fritsch. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
11. Muratani, T., S. Akasaka, T. Kobayashi, Y. Yamada, H. Inatomi, K. Takahashi, and T. Matsumoto. 2001. Outbreak of cefozopran (penicillin, oral cepheps, and aztreonam)-resistant *Neisseria gonorrhoeae* in Japan. *Antimicrob. Agents Chemother.* **45**:3603-3606.
12. Ochiai, S., S. Sekiguchi, A. Hayashi, M. Shimadzu, H. Ishiko, R. Matsushima-Nishiwaki, O. Kozawa, M. Yasuda, and T. Deguchi. 2007. Decreased affinity of mosaic-structure recombinant penicillin-binding protein 2 for oral cephalosporins in *Neisseria gonorrhoeae*. *J. Antimicrob. Chemother.* **60**:54-60.
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. Takahata, S., N. Senju, Y. Osaki, T. Yoshida, and T. Ida. 2006. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* **50**:3638-3645.
15. Whiley, D. M., E. A. Limmios, S. Ray, T. P. Sloots, and J. W. Tapsall. 2007. Further questions regarding the role of mosaic *penA* sequences in conferring reduced susceptibility to ceftriaxone in *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* **51**:802-803.
16. Whiley, D. M., E. A. Limmios, 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.