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Prediction of Decreased Susceptibility to Penicillin of *Neisseria meningitidis* Strains by Real-Time PCR

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Sequence analysis of the *penA* gene, encoding penicillin-binding protein 2 (PBP2), in 30 penicillin-intermediate (PenI) *Neisseria meningitidis* strains showed altered gene sequences due to the translocation of exogenous DNA blocks derived from commensal neisseriae, which are known to have PBP2 proteins with decreased affinity for the antibiotic. In order to obtain a rapid and reproducible method for predicting the PenI phenotype, a real-time PCR assay was set up with primers and probes designed on the basis of the *penA* gene. The A→G mutation at codon 566, in the transpeptidase domain of the *penA* gene (which is present in the whole sample of 30 PenI strains and in all the 41 sequences of PenI meningococci isolated worldwide and has been deposited in the sequence databank), was chosen as a marker of *penA* translocations. Two hybridization probes were designed to distinguish the wild-type *penA* gene in penicillin-susceptible (PenS) meningococci from the mutated *penA* gene at codon 566 in PenI strains. Thermal analysis of probe hybridization revealed a melting temperature difference of at least 6°C between PenI and PenS strains. This real-time PCR protocol characterizes the penicillin phenotype of *N. meningitidis* in a few hours without DNA sequencing and is useful for rapid screening of the penicillin-intermediate genotype among meningococcal isolates.

Neisseria meningitidis, a human pathogen, is a leading cause of life-threatening infections, such as meningitis and sepsis. The treatment of meningococcal infections with penicillin G is still effective, but the number of reports describing the isolation of meningococci with decreased susceptibility to penicillin in different countries is on the rise (4, 6; P. Botha, Letter, Lancet **i:**54, 1988; A. P. Gomes and R. Siqueira-Batista, Letter, Rev. Assoc. Med. Bras. **48:**114, 2002; J. A. Saez-Nieto, J. A. Vazquez, and C. Marcos, Letter, Lancet **336:**54, 1990). This phenomenon is caused by the emergence of *penA* genes whose sequences are altered, determining the production of penicillin-binding proteins (PBPs) with a lower affinity for β-lactam antibiotics. The sequences of these modified *penA* genes are highly variable. As described previously (1), these alterations are due to genetic exchange, in the human nasopharynx, between *N. meningitidis* and naturally resident nonpathogenic neisseriae, resulting in a mosaic structure of the target gene. *penA* sequences from penicillin-intermediate (PenI) strains have been extensively analyzed in different studies (1–3, 12, 13), particularly in the transpeptidase-encoding region (nucleotides 571 to 1746), in order to characterize the genetic polymorphism and to demonstrate a correlation with reduced susceptibility to penicillin. The alteration of only one PBP (PBP2) in *N. meningitidis* suggests that strains with decreased susceptibility are still evolving, so the emergence of penicillin-resistant strains may be expected in the near future, as has been observed for *Streptococcus pneumoniae* (12). The development of molecular methods to monitor the changes in the *penA* genes of meningococci is hence warranted.

This study proposes a reliable and rapid molecular approach to the detection of reduced susceptibility to penicillin in invasive *N. meningitidis* strains. In the absence of a single specific determinant of decreased susceptibility to penicillin in *N. meningitidis*, a real-time PCR assay has been developed for the identification of nucleotide substitutions within the transpeptidase domain of the *penA* gene by oligonucleotide hybridization and thermal analysis. This method permits rapid and reproducible screening of the amino acid substitution Ile_{566}→Val (ATT to GTT). In fact, this mutation has been detected in all 30 PenI strains isolated in Italy and in 41 *penA* sequences of PenI meningococci isolated worldwide and deposited in the National Center for Biotechnology Information website (htpp://www.ncbi.nlm.nih.gov); it has never been detected in penicillin-susceptible (PenS) strains.

MATERIALS AND METHODS

Bacterial strains and penicillin susceptibility test. Five hundred sixteen strains of *N. meningitidis* isolated from patients were received by the reference laboratory at the Istituto Superiore di Sanitá during the period 1995 to 2002. MICs were determined by E-test (AB Biodisk) on Mueller-Hinton agar (Oxoid) supplemented with 5% laked horse blood and incubated in air with 5% CO_2 at 35°C for 24 h. The breakpoints were those recommended for *Neisseria gonorrhoeae* by the National Committee for Clinical Laboratory Standards: for susceptibility, ≤0.06 μg/ml; for resistance, ≥2 μg/ml (8).

For the real-time PCR assay, a panel consisting of 30 PenI strains isolated in different years and 36 PenS meningococci were examined. *N. meningitidis* serogroup B MC58 was used as a susceptible reference strain (Table 1).

DNA isolation, gene amplification, sequencing, and analysis. Chromosomal DNA was extracted by using the QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The coding regions of the *penA* genes (from nucleotides 546 to 1746) of all the strains examined were amplified by using the primers and PCR conditions described by Zhang et al. (15). The PCR products were further purified by use of

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TABLE 1. Characteristics and T_m of 30 penicillin-intermediate and 36 penicillin-sensitive *N. meningitidis* strains

Strain code (accession no. for *penA* sequence)	*N. meningitidis* PenI or PenS phenotype	Yr of isolation	MIC for penicillin (μg/ml)	T_m (°C)
PenI strains				
661 (AJ549315)	C:2b:P1.2	1995	0.125	55.0
759	C:2b:P1.2	1996	0.125	55.0
864 (AJ549316)	B:4:P1.12	1997	0.094	51.7
868 (AJ549309)	B:1:P1.3	1997	0.125	53.5
884	C:2b:P1.5	1998	0.190	55.0
918 (AJ549310)	B:4:P1.16	1999	0.125	53.5
949 (AJ549311)	B:1:P1.15, 16	1999	0.094	53.5
963 (AJ549312)	B:4, 15:P1.16	1999	0.125	53.5
990 (AJ549313)	C:nt:P1.16	1999	0.094	53.5
1000 (AJ549314)	B:14:P1.13	1999	0.190	53.5
1047 (AJ549307)	B:15:nst	2000	0.125	53.5
1067 (AJ549308)	B:15:P1.7, 16	2000	0.190	53.5
1151	C:2b:P1.5	2001	0.125	53.5
1173	W135:nt:nst	2001	0.094	55.0
1194 (AJ549305)	B:4:P1.4	2002	0.125	53.5
1208	C:2b:P1.5	2002	0.190	55.0
1237	C:2b:P1.5	2002	0.125	55.0
1230 (AJ549306)	B:nt:nst	2002	0.094	53.5
1233 (AJ549302)	C:2a:P1.5	2002	0.250	53.5
1234	C:2a:P1.5	2002	0.250	53.5
1228 (AJ549303)	C:2b:P1.5	2002	0.125	55.0
1235	C:2b:P1.5	2002	0.125	55.0
1241	C:2a:P1.5	2002	0.190	53.5
1245	B:15:P1.16	2002	0.250	53.5
1258	C:2b:P1.5	2002	0.250	55.0
1263	C:nt:P1.5	2002	0.125	55.0
1271 (AJ549304)	B:1:P1.6	2002	0.094	53.5
1277	B:nt:P1.16	2002	0.250	53.5
1284	C:2b:P1.5	2002	0.190	55.0
1285	C:2b:P1.5	2002	0.125	55.0
PenS strains				
659	Poli:15:P1.7, 16	1994	0.047	45.5
690	B:2b:P1.10	1995	0.032	45.5
898	B:4, 15:P1.10, 16	1998	0.047	45.5
904	B:21:P1.13	1998	0.012	45.5
921	B:14:P1.13	1999	0.047	45.5
926	B:14:P1.13	1999	0.047	45.5
927	B:14:P1.13	1999	0.047	45.5
939	B:4:P1.4	1999	0.047	45.5
1101	B:15:P1.7, 16	2000	0.032	45.5
1109	B:14:P1.13	2000	0.012	45.5
1120	B:15:P1.4	2001	0.016	45.5
1122	B:14:P1.13	2001	0.008	45.5
1129	B:14:P1.13	2001	0.012	45.5
1130	C:2b:P1.5	2001	0.032	45.5
1133	C:2b:P1.5	2001	0.023	45.5
1135	B:14:P1.13	2001	0.008	45.5
1136	C:2b:P1.5	2001	0.023	45.5
1138	B:nt:P1.4	2001	0.006	45.5
1140	C:2b:P1.5	2001	0.032	45.5
1142	W135:2a:P1.5, 2	2001	0.008	45.5
1147	B:14:P1.13	2001	0.008	45.5
1150	B:14:P1.13	2001	0.008	45.5
1157	B:nt:nst	2001	0.016	45.5
1159	B:15:P1.7, 16	2001	0.008	45.5
1161	B:15:P1.4	2001	0.012	45.5
1175	B:14:P1.13	2001	0.012	45.5
1184	B:14:P1.13	2001	0.080	45.5
1190	W135:nt:P1.3	2001	0.064	45.5
1196	C:2b:P1.5	2002	0.023	45.5
1198	C:2a:P1.5	2002	0.064	45.5
1199	C:2b:P1.5	2002	0.047	45.5
1203	B:2a:P1.5, 2	2002	0.023	45.5
1221	C:2b:P1.5	2002	0.047	45.5
1225	C:2b:P1.5	2002	0.064	45.5
1254	B:15:P1.4	2002	0.016	45.5
1267	B:4:nst	2002	0.047	45.5

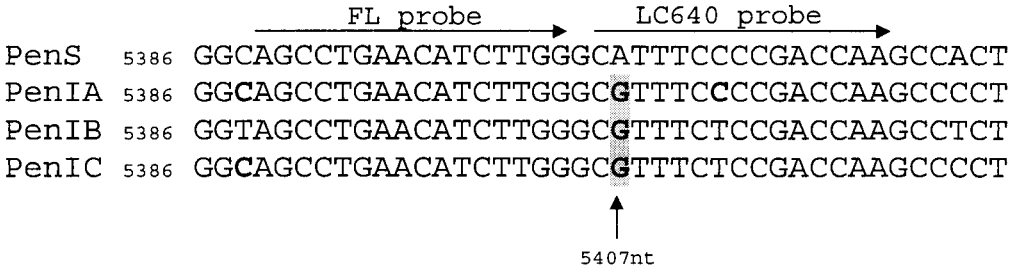

FIG. 1. LightCycler probe positions in a partial *penA* sequence alignment. Nucleotide mismatches in the PenI genotype (*penIA*, *penIB*, *penIC*) are boldfaced.

QIAquick purification columns (Qiagen) for sequencing by the Sanger method (11a) with the ABI Prism DNA sequencer 377 (Perkin-Elmer). The sequences were analyzed with the BLAST program (htpp://www.ncbi.nlm.nih.gov/BLAST/).

Real-time PCR and oligonucleotide thermal analysis. A primer-probe set was designed on the basis of the *penA* gene nucleotide mutations detected in PenI meningococci. The primers for the PCR amplification were primer1 (5′-GATT GTGGCGGTAACCATTGACG; nucleotide positions 5298 to 5320 in EMBL/GenBank accession no. AE002397) and primer2 (5′-CGGGGATATAACTGC GTCCGT; nucleotide positions 5644 to 5624 in accession no. AE002397 [14]), defining a region of about 350 bp. As shown in Fig. 1, the fluorescence probe (FL, donor dye) was designed at positions 5389 to 5404 (5′-AGCCTGAACATCTT GG^{FL}-3′), in a region showing identical *penA* DNA sequences in PenS and PenI strains. The Red640 probe was positioned 1 bp away from the FL probe (5′-$^{Re}{}_{d640}$CGTTTCTCCGACCAA-P-3′) and designed to be homologous to the sequences with accession no. AJ549302 to AJ549315.

The real-time PCR mixture contained 1 μl of purified chromosomal DNA (100 ng), 1 μl (10 pmol) of each primer, 2 μl (2 pmol) of probes FL and LC640, 2.5 μl of $MgCl_2$ (final concentration, 4 mM), 2 μl of Fast-start Master Hybridization Probes reaction mixture (Roche Diagnostic, Mannheim, Germany), and PCR-grade sterile water to a final volume of 20 μl. The samples were run by performing an initial 10-min denaturation step at 95°C followed by 40 cycles of repeated denaturation (10 s at 95°C), annealing (10 s at 48°C), polymerization (10 s at 72°C), and detection of the fluorescence (15 s at 38°C). The last step was added at the end of each PCR cycle to increase red fluorescence levels for the PCR quantitative analysis. The temperature transition rate was 20°C/s in all segments.

Fluorescence was measured in channel 2 at 640 nm. Data were analyzed with LightCycler software (version 5.32) according to the manufacturer's instructions (Roche Diagnostics).

In each run the susceptible reference strain, one PenI strain for which the penicillin MIC was defined, and a negative control (sterile water instead of a DNA template) were included for the reproducibility of results and to check for contamination.

Nucleotide sequence accession numbers. Fifteen representative nucleotide sequences out of the 30 analyzed in the present study were deposited in the EMBL/GenBank nucleotide sequence databases under accession no. AJ549302 to AJ549316 (Table 1).

RESULTS

Susceptibility to penicillin and *penA* sequence analysis. Strains with PenI phenotypes account for more than 10% of all meningococci isolated in Italy from 1995 to 2002. Interestingly, during the year 2002, a higher proportion of PenI meningococci was detected (26%), and the C:2b:P1.5 phenotype was predominant. Moreover, MICs for PenI strains were higher than those found in previous years (7). The phenotypic characteristics of all the strains examined are shown in Table 1.

The amino acid change from Ile_{566} to Val_{566} (ATT→GTT transition), found at the 3′ end of the *penA* gene in all the PenI meningococci examined, allowed us to design the probes used in the real-time PCR assay (Fig. 1). Four other nucleotide transitions (causing amino acid substitutions Phe_{504}→Leu, Ala_{510}→Val, Ile_{515}→Val, and His_{541}→Asn), derived from commensal neisseriae, were always associated with the Ile_{566}→Val mutation. The mutation at codon 566 was chosen as a marker for *penA* translocation, since the other four mutations were localized within an extremely variable region that was not useful as a target sequence for primer hybridization and thermal analysis. The five mutations were also consistently detected in all 41 sequence alignments of PenI *N. meningitidis* strains deposited by other authors (htpp://www.ncbi.nlm.nih.gov).

LightCycler *penA* mutation assay. Oligonucleotide hybridization and thermal analysis, using the FL and LC640 probes, yielded different melting curves for susceptible *N. meningitidis* strains (45.5°C) and PenI strains carrying the ATT_{566}→GTT mutation, which resulted in a melting temperature (T_m) increase of 6 to 9.5°C. Seventeen PenI strains that carried the mutation at codon 566 showed a T_m of 53.5°C (PenIB), whereas 12 PenI strains that also had a C→T transition showed a T_m of 55°C (PenIC) (Fig. 2). The presence of this nucleotide difference localized 1 base before the FL probe results in a 1.5°C increase in the thermal dissociation of the FL-LC640 probes. The fact that differences in neighbor bases can cause a slight change in the stability of the probes has been reported previously (10). One strain harboring the mutation at codon 566 also carried an additional nucleotide mismatch, a C instead of a T, compared to the LC640 probe sequence (Fig. 1) and showed a T_m corresponding to 51.7°C (PenIA) (Fig. 2). In summary, all PenI strains, showing the codon 566 mutation alone or in combination with other nucleotide substitutions, produced discernible peaks in the range from 51 to 55°C, allowing them to be distinguished from the PenS strains, which peaked at 45.5°C (Fig. 2). Results obtained by the real-time PCR assay were reproducible and always in agreement with the nucleotide sequencing data and MICs (Table 1). In fact, all PenI strains showed T_m values between 51.7 and 55°C, whereas all PenS strains showed a T_m of 45.5°C.

DISCUSSION

Since the introduction of penicillin for the treatment of meningococcal infections, the occurrence of *N. meningitidis* strains resistant to penicillin due to β-lactamase-producing plasmids has been rare (4), whereas the frequency of meningococci with decreased susceptibility to the antibiotic has steadily increased (5, 11; Saez-Nieto et al., letter). This behavior is due to the well known lower penicillin affinity of the PBP2 encoded by DNA originating from nonpathogenic com-

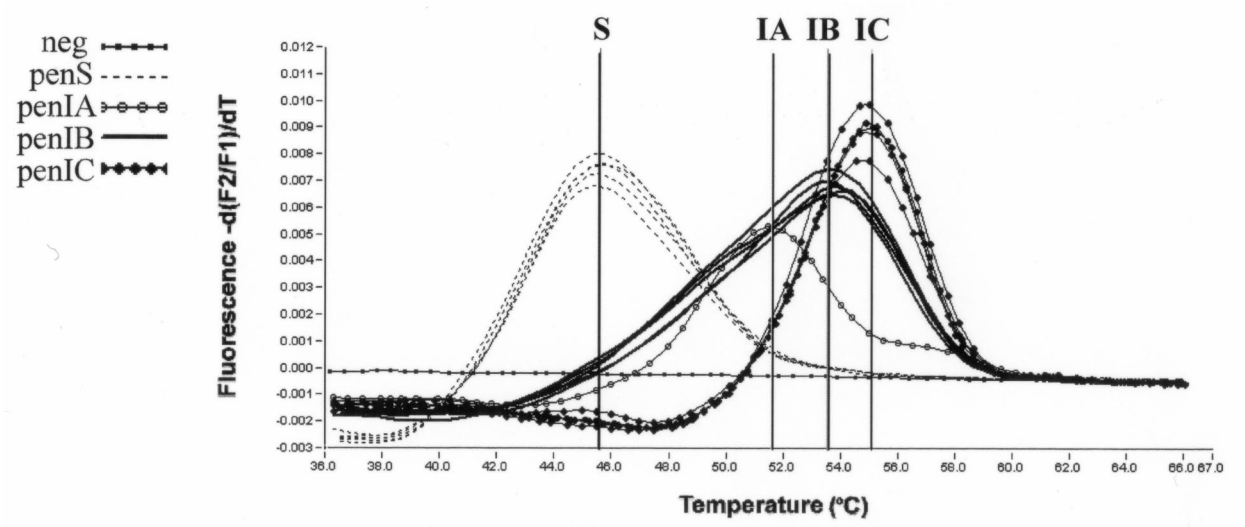

FIG. 2. T_m curves for three different PenI genotypes (*penIA*, *penIB*, and *penIC*). All PenS strains peaked within the same curve. Melting peaks were derived by plotting the negative derivative of fluorescence with respect to temperature ($-dF/dT$).

mensal nasopharyngeal neisseriae, but the possible role of other genetic mechanisms cannot be entirely ruled out (9).

Surveillance studies have been conducted in Italy since 1994, and a changed pattern of penicillin susceptibility among circulating meningococci during the year 2002 has been observed. In fact, a greater number of PenI strains were detected for which MICs were higher than those found in previous years. A unique clone, C:2b:P1.5, belonging to the ST8/A4 cluster, accounted for 38% of PenI strains isolated in 2002, marking a change in the situation previously observed in Italy (7).

The development of molecular approaches for the prediction of meningococcal susceptibility to penicillin is particularly useful for rapid screening and for the achievement of good interlaboratory standards.

Studying the sequence alignments of our PenI strains, we found the presence of five different amino acid substitutions, derived from exogenous DNA, at the 3′ end of the *penA* gene. Interestingly, these substitutions were detected in all the *penA* genes sequenced from PenI meningococci, including those deposited by other authors in GenBank. The real-time PCR assay that we subsequently developed allows one to look for a single genetic target in the prediction of different penicillin susceptibility phenotypes in *N. meningitidis*. One of the five substitutions had a nucleotide sequence favorable for the design of probes for the real-time PCR in a LightCycler assay. One of the two probes harbors this mutation at the second nucleotide position and successfully identified meningococci as intermediate in agreement with MICs higher than 0.06 μg/ml. Three different melting profiles were provided within the PenI phenotype due to other mismatches in the *penA* gene sequence. In all cases, the T_m difference between a susceptible and an intermediate strain ranged from 6 to 9.5°C, resulting in discernible and easily interpretable results.

In conclusion, this real-time PCR protocol makes it easy to detect and characterize the penicillin phenotypes of meningococci in a few hours without DNA sequencing. The reproducible results in the detection of penicillin-intermediate *N. meningitidis* strains underline the reliability of this assay for discriminating among meningococcal isolates. Thus, it could be an extremely useful tool for those long-term monitoring programs which already exist in several countries.

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