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High-Resolution Melt Curve Analysis for Identification of Single Nucleotide Mutations in the Quinolone Resistance Gene *aac(6′)-Ib-cr*[▽]

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We have developed a simple PCR-based high-resolution melt curve analysis for identification of the quinolone resistance gene *aac(6′)-Ib-cr* through regions encompassing the two defining single nucleotide mutations. Dissociation curves showed 100% concordance with DNA sequencing, including the identification of a strain where *aac(6′)-Ib* and *aac(6′)-Ib-cr* coexist.

The *cr* variant of *aac(6′)-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin and norfloxacin by N acetylation of their piperazinyl amines (8). *aac(6′)-Ib-cr* belongs to the group of plasmid-mediated quinolone resistance genes that determine small increases in the MICs that are sufficient to facilitate the selection of higher-level-resistance mutants (10). However, this low-level quinolone resistance is below the CLSI breakpoint for nonsusceptibility and is not detected in the clinical laboratory. Development of efficient techniques for detection of clinical isolates carrying *aac(6′)-Ib-cr* may improve optimization of antibiotic treatment. The resistance phenotype of AAC(6′)-Ib-cr is dependent on the effects of the individual mutations. Asp181Tyr (coded by G541T) produces a partial-resistance phenotype and Trp104Arg (coded by either T310C or T310A) no detectable resistance, but together, the two mutations confer the full resistance phenotype (the nucleotide positions correspond to GenBank accession number AF479774; see also the cr variant under GenBank accession number AY259086) (8). We previously showed that the gap-ligase chain reaction (LCR) is an inexpensive technique suited to large-scale surveys, albeit time-consuming (13).

Improved real-time PCR machines and software analysis packages in recent years have enhanced the resolution of melting temperature (T_m) differences between amplicons from 2°C to 0.01°C in modern instruments (4, 7). Such resolution is now sufficient for identification of transitions or transversions that involve A ↔ C or G ↔ T and can be applied in high-resolution melting curve analysis (HRMA) to identify a single nucleotide change inside a full-length amplicon (12). Melt analysis and its more sophisticated high-resolution variant are being increasingly applied to identify quinolone resistance mutations in type II topoisomerases of *Haemophilus influenzae* (5) and *Neisseria gonorrhoeae* (11), to genotype *Mycoplasma pneumoniae* isolates (9), or to identify multidrug-resistant *Mycobacterium tuberculosis* (6). We developed and validated a real-time PCR-based HRMA using SYBR green I to rapidly detect *aac(6′)-Ib-cr* and distinguish it from *aac(6′)-Ib*.

A homology search in GenBank identified 181 sequences described as *aac(6′)-Ib*, of which 22 corresponded to the *aac(6′)-Ib-cr* variant. Alignment of all sequences was used to design two pairs of primers (Geneious Pro 4) (2). The pair comprising aac6-5′278 (GTCGTACGTTGCTCTTGGAA) and aac6-5′352 (GGTCTATTCCGCGTACTCCT) and the pair comprising aac6-3′508 (GGGTTTGAGAGGCAAG GTA) and aac6-3′582 (GAATGCCTGGCGTGTTTG) amplified 73- and 74-bp products, designated the 5′ region and the 3′ region, respectively, that corresponded to nucleotides 278 to 352 and 508 to 582, respectively, in *aac(6′)-Ib* (GenBank accession number AF479774).

In developing the HRMA method, we used four different alleles of *aac(6′)-Ib* that we had previously generated by site-directed mutagenesis (8): aac0, encoding wild-type *aac(6′)-Ib*; aac1, encoding *aac(6′)-Ib-cr*; and aac2 and aac3, encoding *aac(6′)-Ib* with single mutations T310C and G541T, respectively. The assay was validated on nine *aac(6′)-ib-cr*-positive and 10 wild-type strains from a collection of clinical isolates already screened for *aac(6′)-Ib-cr* by gap-LCR and verified by sequencing (13).

Plasmid DNA from control strains was extracted using a QIAamp DNA minikit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Colonies were transferred to Tris-HCl (pH 7.4) in a 2-ml screw-cap tube and heated for 2 min at 98°C to prepare DNA templates from tested strains.

Real-time PCR and HRMA were performed using a Rotor-Gene 6000 apparatus (Corbett Life Science, Australia) in a total volume of 20 μl; the run consisted of 30 cycles at 93°C for 10 s, followed by 58°C for 10 s and 72°C for 6 s. The high-resolution-melt (HRM) conditions were 2 s at 95°C followed by 90 s at 55°C premelt, with an HRM ramp from 76°C to 86°C, rising by 0.04°C each step and holding for 2 s on each step. Gain optimization before the melt on all tubes was selected. SYBR green I (DyNAmo Flash SYBR green quantitative PCR [qPCR] kit; Finnzymes) was used with an excitation wavelength at 470 nm and detection at 510 nm. For normalization, the temperature ranges were 75.34°C to 77.51°C for the leading range and 82.39°C to 84.30°C for the trailing range. Calcula-

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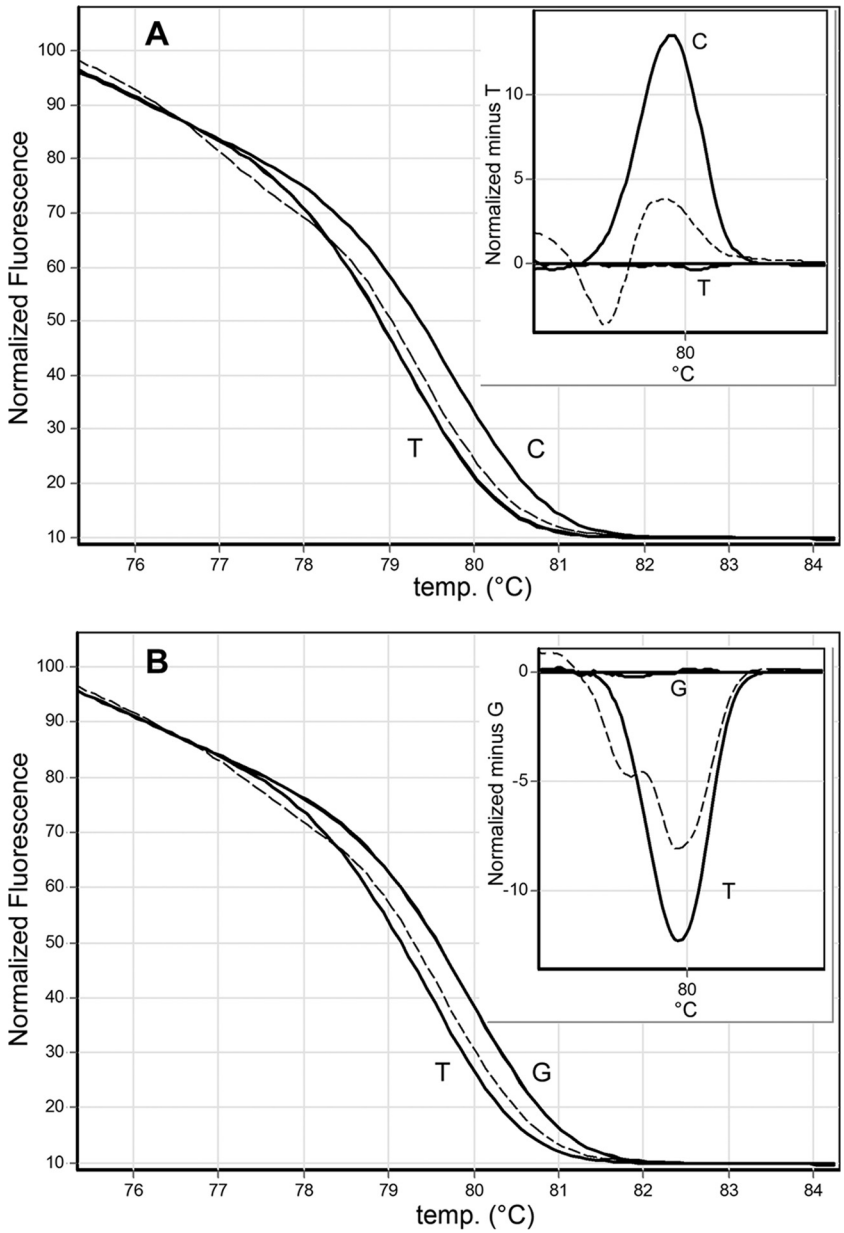

FIG. 1. Dissociation curves. Normalized and temperature-shifted difference plots for mutant discrimination by HRMA. (A) Normalized melt curve plot of the 5′ region showing T → C transition and (as shown in the nested graph) the normalized temperature minus the temperature shift for the same amplicon (T). (B) Normalized melt curve plot of the 3′ region, showing G → T transversion and (as shown in the nested graph) the normalized temperature minus the temperature shift for the same amplicon (G). Corresponding nucleotides (C, G, and T) are depicted next to each curve. The nucleotide present in *aac(6′)-Ib* was used to normalize each temperature shift graph. Dotted lines correspond to a heterozygote.

tions were done using the Rotor-Gene software program (version 1.7). A confidence value is provided as an integrity check of autocalled results. Serial 10-fold dilutions of extracted DNA from the control strains were amplified and subjected to HRMA. The distinctive typing that resulted from the type-specific melt profiles of the 5′ region (Fig. 1A) and the 3′ region (Fig. 1B) showed that all amplicons were reliably sorted into one of the two distinct groups within each region.

The estimated error rates for genotyping homozygotes as a function of their T_m varied from instrument to instrument, and this rate was found to be less than 0.01 at 0.5°C for an amplicon of 110 bp with the use of the Rotor-Gene 3000 instrument (4). The intra-assay variation was calculated for two replicas of four 10-fold dilutions of each mutant. The standard deviations (SD) of the T_m varied from 0.01 (with a T_m of 79.20°C for nucleotide T in the 5′ region and a 73-bp amplicon) to 0.055 (for nucleotide T in the 3′ region with a T_m of 79.26°C and an amplicon of 74 bp). The differences in T_m (ΔT_m) between amplicons for the 5′ and 3′ regions were 0.62°C and 0.71°C, respectively, with a confidence level above 97% in all cases.

As expected, no PCR products were obtained from dozens of clinical strains lacking *aac(6′)-Ib* and its variant. We then

assayed 43 carbapenemase-producing *Enterobacteriaceae* with an unknown *aac(6′)-Ib* genotype isolated from wounds, sputa, and urine samples. One and 41 isolates were positive for *aac(6′)-Ib-cr* and *aac(6′)-Ib*, respectively; the HRMA results for these amplicons were sorted in the expected group, with confidence averages of 97.9% for the 5′ region (SD = 2.3) and 97.1% for the 3′ region (SD = 2.1). For one strain, a dissociation curve was interpreted as having variations (less than 85% confidence) in both the 5′ and the 3′ regions (Fig. 1). The distinct T_m plot of this amplicon is visible on the normalized graphics (Fig. 1, nested graphics). Sequencing demonstrated double peaks corresponding to the nucleotides cytosine and thymine at position 310 and guanine and thymine at position 541 in *aac(6′)-Ib-cr*.

PCR products were obtained for every strain used for validation, thus supporting the utility of the boiling extraction method as a reliable, fast, and inexpensive method for obtaining whole-cell DNA as a template for this PCR.

HRMA may detect other mutations that are not the target of the screening within the amplified region. In addition, because the melting temperature is the same, the HRMA might not have detected a T310A mutation. Recently, a report on the detection of *aac(6′)-Ib-cr* through its T310C or T310A mutations by the use of an asymmetric concentration of primers to promote amplification of the DNA strand complementary to an unlabeled and 3′ phosphorylated probe for HRMA was published (1). An evolutionary step for *aac(6′)-Ib* with a single mutation in either position 310 or position 541 (not investigated in that study) is plausible (1, 3), and its identification would be an important contribution to the understanding of the evolution of *aac(6′)-Ib-cr* and the tracking of its epidemiology. By analyzing the two *aac(6′)-Ib-cr*-characterizing regions, we ensured the accuracy of detection of the cr variant, even indicating whether this variant had been determined by a mutation at position 541. We have developed a simple and rapid real-time PCR-based HRMA that is able to detect *aac(6′)-Ib-cr* and discriminate between the two *aac(6′)-Ib* single nucleotide mutations required for the ciprofloxacin resistance phenotype. This approach provides an improvement over laborious procedures such as gap-LCR or expensive sequencing methods. Further research is needed in applying rapid diagnostic procedures for the detection of additional plasmid-mediated quinolone resistance genes.

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