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Identification of *P1* Variants of *Mycoplasma pneumoniae* by Use of High-Resolution Melt Analysis[▽]

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***Mycoplasma pneumoniae* is a leading cause of community-acquired pneumonia. Although two genetically distinct types of *M. pneumoniae* are known, variants of each also exist. We used a real-time PCR high-resolution melt genotyping assay to identify clinical variants which may provide greater insight into the genetic distribution of *M. pneumoniae* strains.**

Mycoplasma pneumoniae is a pervasive pathogen that accounts for up to 20% of all community-acquired pneumonias (14, 15). Although its genome is highly homogeneous between strains, the *P1* gene, which encodes an immunogenic adhesion protein, serves as a target to categorize isolates into either a type 1 or a type 2 genetic group (3). Numerous reports of genetic variants of each type have also been described (2–4, 6, 8). Spuesens et al. recently showed that intragenomic homologous DNA recombination occurs within the RepMP2/3 and RepMP4 regions of the *P1* gene (MPN141) of *M. pneumoniae* and strain differences can be attributed to variation within RepMP elements (13). Dumke et al. also reported that *M. pneumoniae* variants differ only within the RepMP2/3 element (5). The clinical relevance of these findings is unclear, but epidemiological evidence of temporally based type-specific immunity in the population suggests these variations impart an advantage to the organism (6, 11). Moreover, the well-documented 3- to 7-year cyclic patterns of population-based, type-specific outbreaks seem to support this hypothesis (7, 9, 11).

Historically, typing schemes were based upon restriction fragment length polymorphism (RFLP) analysis of digested PCR products of the *P1* gene; however, Degrange et al. recently described a multiple-locus variable-number tandem-repeat analysis assay that was able to sort 265 strains into 26 distinct groups (1). This assay requires amplification of five loci followed by capillary electrophoresis and analysis but provides a greater level of genomic resolution. We recently reported the development of a real-time PCR assay that is able to rapidly distinguish *M. pneumoniae* isolates into type 1 or type 2 categories (12). We used this technique to classify 102 isolates using real-time PCR followed by high-resolution melt (HRM) analysis of a variable region partially spanning the RepMP2/3 element of the *P1* gene (10, 12). The current study reports the use of this assay to identify variants of each group based upon intratype sequence deviations. Through the analysis of distinct HRM profiles from clinical isolates obtained by the CDC Respiratory Diseases Branch, we have been able to identify variants of each type. In this report, we describe the identification, sequence analysis, and unique HRM profiles of three diverse *P1* variants (isolates 3, 684, and 549) and demonstrate the utility of this assay for detecting *M. pneumoniae* variants along with prototypical type 1 and 2 strains.

M. pneumoniae culture and isolation were performed as previously described (16). M129 (ATCC 29342) and FH (ATCC 15531) were used as the reference strains for type 1 and type 2, respectively. Nucleic acid from all isolates was isolated using the QIAamp DNA mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RFLP analysis, PCR-HRM, and sequencing of the ~1.9-kb *P1* gene fragment were performed as previously described (12). All real-time PCR-HRM assays were performed in triplicate.

The HRM profiles for each reference strain and the variant isolates are shown in Fig. 1A ("normalized" mode) and B ("difference" mode). The melt curves for M129 and FH are clearly distinct from each other, as previously reported (12). The HRM melt profiles of each variant in Fig. 1A display unique curves which do not match those of either reference strain. Isolates 684 and 549 appear to share greater similarity with the HRM profile of type 1, as each consistently melts at a higher temperature than FH (type 2). The dissociation curves of these variants (and true type 1 strains) are shifted to the right, while isolate 3 appears to resemble more of a type 2 strain, as evidenced by a lower melting temperature similar to that of FH. These unique profiles are also readily apparent when viewed in the "difference" mode whereby FH is normalized to be the "standard" and M129 and each variant display a distinct HRM curve (Fig. 1B). Furthermore, RFLP analysis of isolates 684 and 549 reveal a type 1 digestion profile whereas isolate 3 displays a type 2 pattern (data not shown). These findings underscore the superior discriminatory nature of the current assay compared to RFLP methodology.

To further explain these observations, we sequenced the ~1.9-kb P1 amplicons of these isolates and compared them to those of each reference strain. The alignments of isolates 684 and 549 with M129 showed two base changes within the amplicon of each variant; however, these changes were not identical (data not shown). Interestingly, variant 684 contained two transitions, one at position 384 of the amplicon (G→A) and the other at position 1223 (C→T); however, variant 549 pos-

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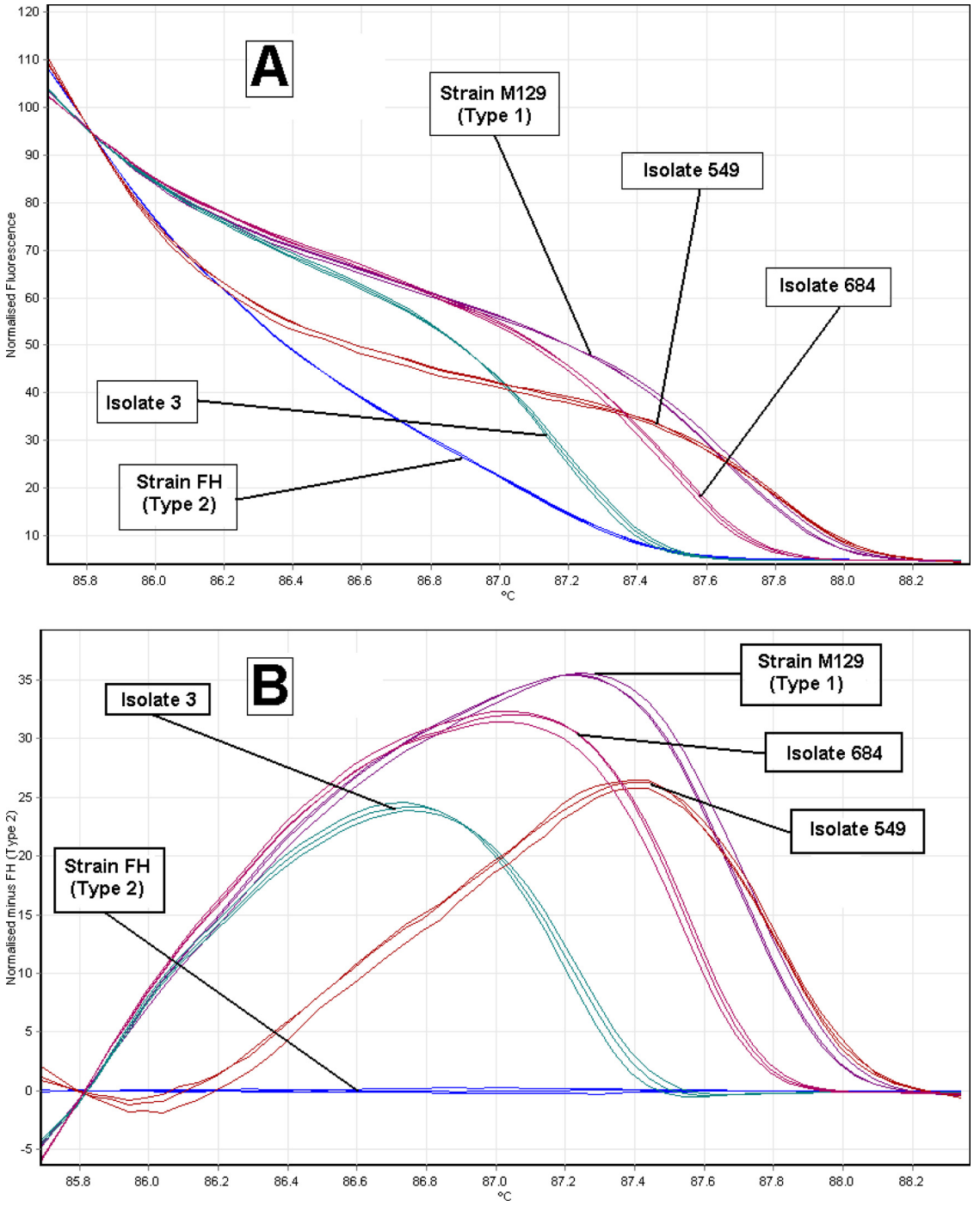

FIG. 1. (A) HRM normalized mode graph. HRM of reference strains FH (type 2) and M129 (type 1) and type 1 P1 variants 684 and 549 and type 2 P1 variant 3. Strains were tested in triplicate. (B) HRM difference mode graph. All strains are "normalized" to the FH (type 2) reference strain profile to more clearly show deviations. Strains were tested in triplicate.

sessed both a transversion (C→A) and a transition (A→G) very close to one another at loci 820 and 822, respectively. These changes in base composition and location likely contribute to thermodynamically distinct melting domains which influence their individual dissociation curves (17). The sequence data from isolate 3 are shown in Fig. 2. Sequence analysis of this variant compared to those of both M129 and FH reveal extensive differences throughout the amplicon; however, similarities to both type 1 and 2 sequences also exist. This alignment also reveals a considerable amount of sequences unique to isolate 3 at this locus. Collectively, these data suggest that isolate 3 exhibits an "intermediate" genotype of type 1 and 2 at this locus. Despite these variations, isolate 3 is classified as a type 2 by PCR-RFLP analysis, thus lending itself to be more aptly described as a "type 2 P1 variant" (12).

This study describes the use of a specific real-time PCR-

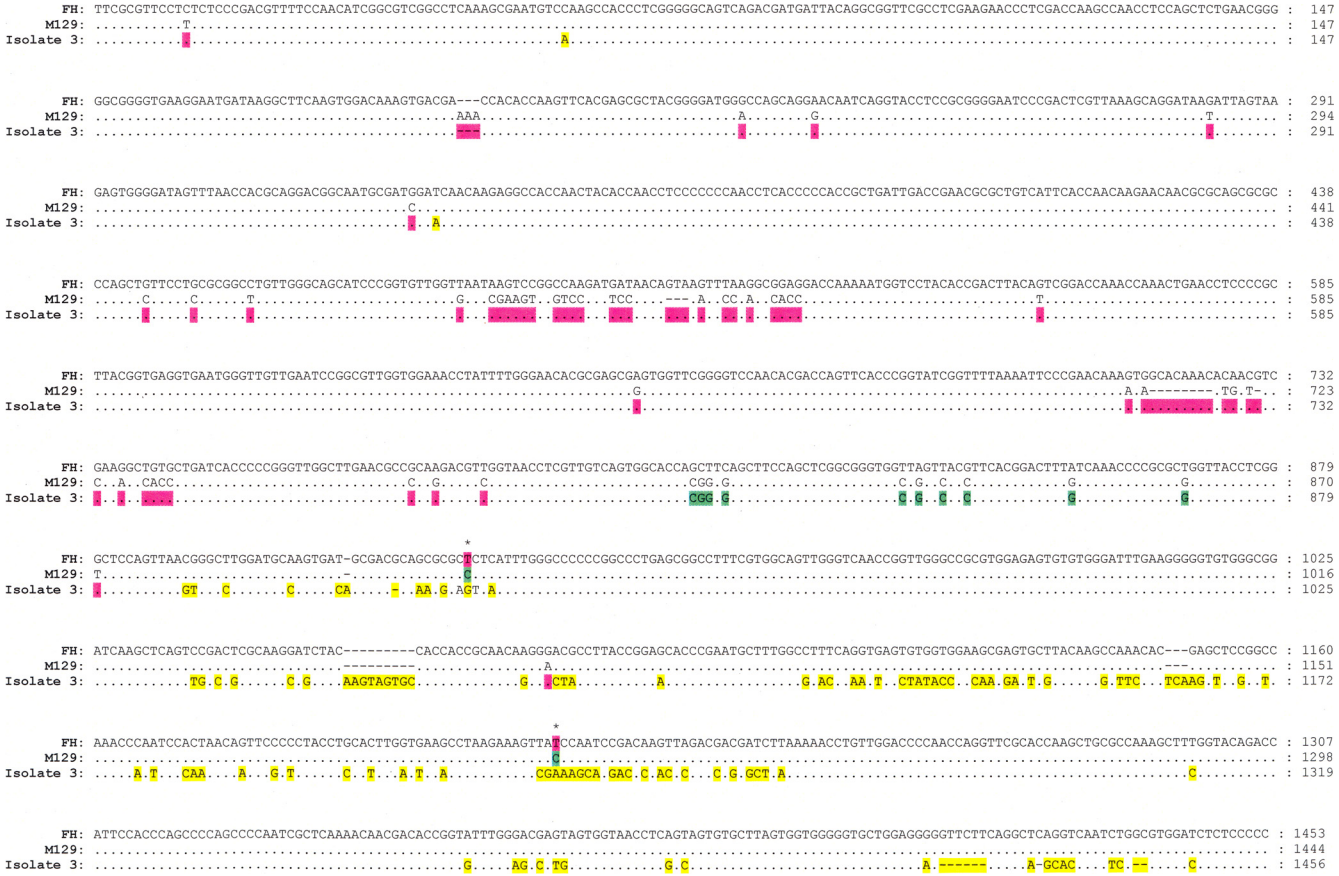

FIG. 2. Sequence alignment of the variable region of reference strains FH (type 2), M129 (type 1), and isolate 3. The FH strain is shown as the consensus sequence. Isolate 3 has type 1- and type 2-specific similarities (shown in green and pink, respectively), as well as unique sequences (shown in yellow). An asterisk denotes variation in all three strains.

HRM assay that can be used to provide greater discriminatory capability for typing and characterizing isolates of *M. pneumoniae* beyond merely two genomic groups. We report the identification of clinically relevant *M. pneumoniae* variants using this rapid (<2.5 h), highly reproducible closed-tube method. Unfortunately, because these variants seem to exist at lower frequency within the population than prototypical type 1 or 2 isolates, it is difficult to determine the overall reproducibility of this test. Our findings also demonstrate the utility of this technology on longer (>500-bp) amplicons containing minor changes (17). By targeting the immunodominant and variable *P1* gene, this assay may be a particularly valuable genetic screening tool for phylogenetic, epidemiological, and immunological studies. Identification of *M. pneumoniae P1* types may reveal epidemiological trends, such as host preference factors (i.e., age, gender, and race, etc.) or temporal or geographical tendencies or shifts, as well as pathogenicity observations (i.e., clinical presentation). The current assay may also aid in further delineating and characterizing the genetic profiles and strain variations of *M. pneumoniae* circulating in the population and complement the existing typing procedures to provide greater discriminatory power.

Nucleotide sequence accession numbers. GenBank accession numbers for the *P1* amplicons of variants 3, 684, and 549 are GQ861494, GQ861493, and GQ861495, respectively.

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