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Microbiological Applications of High-Resolution Melting Analysis

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High-resolution melting (HRM) analysis uses real-time PCR instrumentation to interrogate DNA sequence variation and is a low-cost, single-step, closed-tube method. Here we describe HRM technology and provide examples of varied clinical microbiological applications to highlight the strengths and limitations of HRM analysis.

The term "high-resolution melting" (HRM) analysis encompasses DNA fragment analysis techniques that involve the accurate determination of the relationship between temperature and extent of denaturation. First described by Carl Wittwer's group for clinical applications (6, 8), HRM is typically performed with a real-time PCR instrument, immediately after the PCR. HRM is often used to interrogate specific single nucleotide polymorphisms (SNPs) but can also be applied to SNP discovery, the discrimination of alleles defined by multiple SNPs or tandem-repeat number, and the determination of DNA methylation status. The advantages of HRM technology are its low cost, use of generic instrumentation available in many laboratories, and the simplicity of the approach—with the great majority of applications effectively incorporating single-step and "closed-tube" methods.

While whole-genome sequencing is likely to supersede many microbial genotyping methods, the speed and simplicity of HRM and its generic technology platform suggest that microbiological analysis protocols that incorporate HRM for first-pass screening may have considerable utility. Indeed, as high-throughput sequencing improves the knowledge of specific mutations correlated to important microbiological phenotypes, there will be more targets for HRM-based assays. The growing popularity of HRM for microbiological applications is reflected by the 125 publications in this field since 2006, with 59 publications in the past 18 months alone (to 8 June 2012). Here we briefly describe HRM technology and provide examples of clinical microbiological applications to highlight the strengths and limitations of HRM.

HRM THEORY AND TECHNOLOGY

The denaturation of a DNA fragment with increasing temperature defines the "melting curve." This is usually sigmoidal in shape (Fig. 1), and the melting temperature (T_m) can be calculated by determining the peak of the melting curve first derivative (dF/dT). The T_m is positively correlated with sequence length and percent GC content (%G+C). An approximation of the relationship between sequence content and length is as follows: $T_m = 81.5 + 16.69 \times (\log[Na^+]) + 0.41 \times (\%G+C) - (500/\text{sequence length})$. The correlation of T_m with %G+C is due to the additional hydrogen bond between GC pairs compared with AT pairs. According to the formula given above, the T_m change (ΔT_m) corresponding to a single G-C ↔ A-T change is (41/sequence length) in °C. We have extensive experience with the Corbett Rotorgene device, and in our hands the limit for robust ΔT_m discrimination is approximately 0.2°C. This is the ΔT_m predicted from a single G-C ↔ A-T change in a 205-bp sequence. Unsurprisingly, our experience is that 200 bp is the approximate length limit for robust T_m-based detection of such changes (see Fig. 1E for an example where variants representing a single G+C difference are not reliably discriminated). For smaller fragments, single-base-pair changes of the %G+C content generally have predictable effects on the T_m that are consistently detected (Fig. 1) (11). However, for any given %G+C value, the increase in T_m with length is asymptotic, with little increase beyond approximately 100 bp. This can have the counterintuitive consequence that alleles that differ on the basis of length, such as variable-number tandem repeats, can be difficult to resolve by HRM analysis if the repeat number variations do not change the %G+C and the amplimer is of appreciable size (22). HRM analysis also makes use of the precise melting curve shape, which is a function of the actual DNA sequence. For example, if regions of the sequence have different %G+C contents, this can result in bi- or multiphasic melting curves, which can assist in allele discrimination (Fig. 1B) (11). Methods using nearest-neighbor thermodynamic models of DNA melting have been developed to facilitate the prediction of such melting domains (5). Nonetheless, we have found that experimental validation of predictions continues to be required.

For detection of PCR products, real-time PCR devices use fluorescent molecules—either probes that fluoresce following hybridization with the PCR product or dyes such as SYBR green that fluoresce only when complexed with double-stranded nucleic acid. HRM analyses most often entail the use of a DNA binding dye in an HRM-capable real-time PCR device. Real-time PCR instruments from the major manufacturers, including Applied Biosystems, Roche, and Qiagen, have HRM capabilities—principally the ability to control the temperature very accurately and to perform automated HRM curve comparison. The melting curve is, in effect, a spectrum that is analyzed using a model-free technique on the basis of shape, rather than being used only to calculate the T_m. Variants forms of HRM analysis include conditional fluorescence probe-based methods, which reduce the size of the region being analyzed to a subset of the amplimer, and the assessment of DNA methylation status.

Although examples of the first generation of real-time PCR devices using DNA binding dyes such as SYBR green were able to generate melting curves and allow T_m calculation, in general, the accuracy and resolution were regarded as insufficient to discrim-

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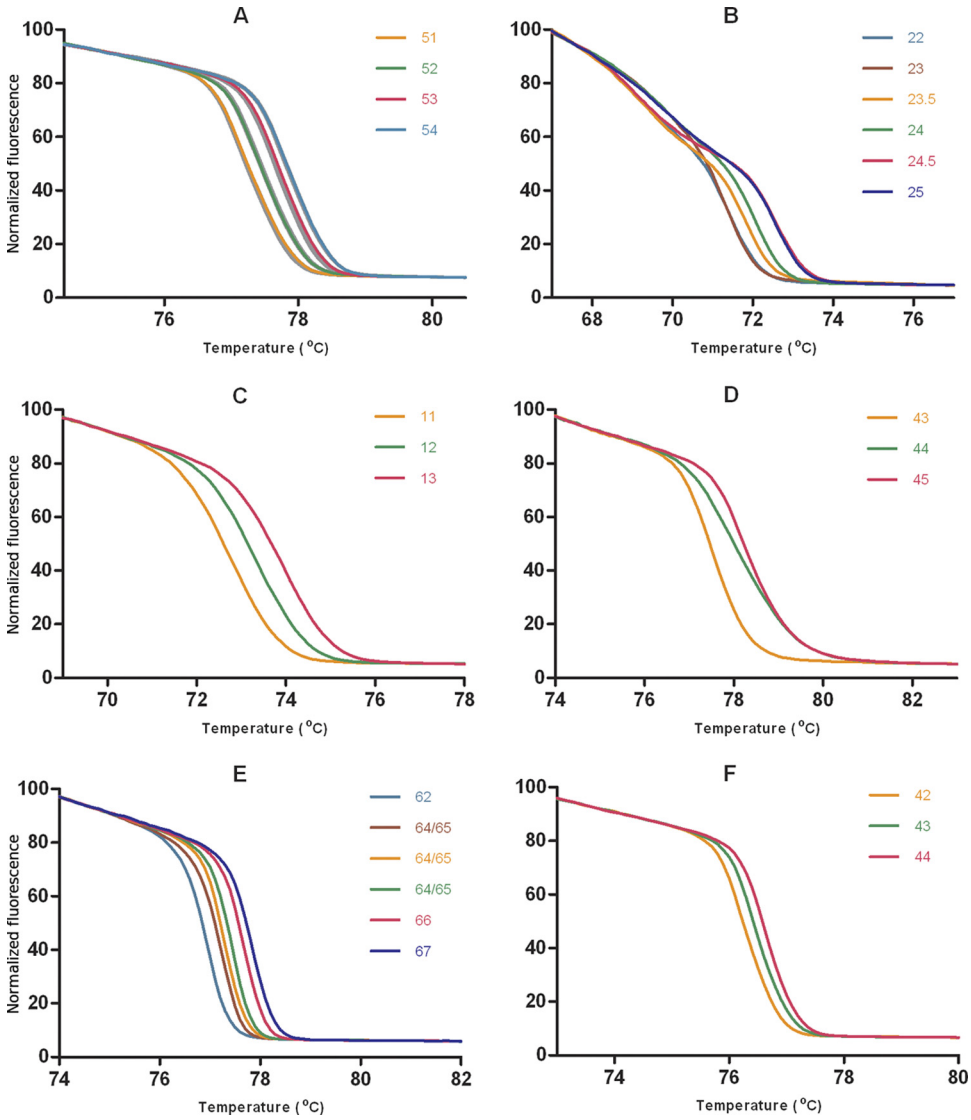

FIG 1 High-resolution melting curves for six internal fragments from the multilocus sequence typing loci of *Staphylococcus aureus*. The number of G+C residues contained in the fragment labels the curves. For example, in panel A, the melting curve variants of a 181-bp internal fragment of *arcC* demonstrate an increasing melting temperature (T_m) as the number of G+C residues increases from 51 to 54. In panel B, there is evidence of two melting domains in this 140-bp internal fragment of *aroE*, resulting in the ability to discriminate variants of the same %G+C. These additional curves have been labeled 23.5 and 24.5. In panel E, melting curves from variants with 64 and 65 G+C residues could not be consistently discriminated for this large 219-bp internal fragment of *tpi* and have been labeled 64/65. The other fragments are from *gmk* (C), *pta* (D), and *tpi* (F) (a region of *tpi* different from that represented in panel E). (Reproduced from reference 11.).

inate allelic variants. "Saturating" double-stranded nucleic acid-specific fluorescent dyes (e.g., LCGreen, SYTO9, EVAGreen) have been developed that can be used at concentrations that result in saturation of all DNA binding sites, without problematic inhibition of the PCR. In contrast, SYBR green is highly inhibitory of PCR at saturating concentrations. With heterozygous samples, saturating dyes may enhance the differences in the melting curve that are conferred by sequence variation and better identify homozygous and heterozygous samples. The mechanistic basis for the inferior performance of nonsaturating dyes is not entirely clear but has been previously reported to be associated with the potential for nonsaturating dyes to redistribute during melting, thus damping the reduction of fluorescence in the early stages of melting (26). Where the detection of heteroduplexes is not required, as is usually the case with prokaryotes, we have found that nonsaturating dyes such as SYBR green approach the effectiveness of saturating dyes (15).

APPLICATIONS OF HIGH-RESOLUTION MELTING ANALYSIS

Species identification of microorganisms. HRM analysis of ribosomal genes or other highly conserved genes has been used extensively to identify microorganisms to the species level. One of the earliest reported applications of HRM was the identification of species within the *Mycobacterium chelonae-M. abscessus* group (13). No universal HRM-based method for bacterial identification has been reported, with published methods focusing upon particular genera (17), particular classes of species such as biothreat agents (27), or species most likely to be encountered in sepsis (14).

As an example of fungal species identification, HRM analysis was used to discriminate between *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans*, and *Cryptococcus gattii* species by interrogating a 100-bp fragment of internal transcribed spacer 1 from ribosomal DNA (7). The accurate detection and identification of mucormycetes from both culture and tissue samples have obvious rapid diagnostic utility (9).

Genotyping within microbial species. The epidemiological tracking and monitoring of microorganisms currently require high-throughput low-cost molecular typing methods. One extensively used class of typing methods is single-locus sequence typing based on hypervariable gene fragments. Such hypervariable genes are potentially amenable to HRM analysis. This has been demonstrated with the *Campylobacter jejuni* CRISPR and *fla* loci (12) and the *S. aureus spa* locus (19, 22). Those studies demonstrated that HRM can resolve significant numbers of alleles of individual loci. Single-locus HRM analysis has also been applied to virus genotyping, with one study of the fowl adenovirus demonstrating useful HRM analysis of a remarkably large 897-bp fragment that produced HRM curves consistent with multiple melting domains (18).

Multilocus HRM-based microbial genotyping methods have also been developed. These have the potential to provide better resolving power, particularly in analysis of recombinogenic species with incomplete linkage between allelic states. Five genes were targeted in the varicella-zoster virus genome, and the combination of SNPs discriminated the key genotypes, including the V-Oka vaccine strain (20). A generalized multilocus approach to bacterial typing, "Minim typing," is based upon HRM analysis of fragments internal to the fragments used for multilocus sequence typing (MLST). The fragments are selected to encompass SNPs that in combination provide optimized resolution as measured by the Simpson's Index of Diversity. The fragments also contain "bystander" SNPs that increase the number of HRM alleles of the fragments and the resolution of the method (1, 11, 16, 23). Interrogating six regions from the MLST loci resolves known *S. aureus* STs into 268 "melting types" (Fig. 1) (11). Similarly, *Enterococcus faecium* can be resolved into 231 melting types (23). Although HRM methods do not have the resolving power of MLST or whole-genome sequencing, they are rapid and consist of a single step, require only generic instrumentation, and provide digitizable results. In the era of low-cost whole-genome sequencing, a rational workflow for microbial surveillance could incorporate an HRM-based method for the first-stage genotyping, with whole-genome sequencing as required.

An interesting application of HRM technology has been to determine HIV diversity within a single host (3, 24). With increasing diversity of sequence variation within an amplicon, the melting of the duplexes present in a sample occurred over a broader temperature range, as measured by the width of the dF/dT curve (24). Diversity at regions in *gag*, *pol*, and *env* was found to be higher in samples from nonrecent infection than in those from recent infection as well as in mothers than in their infected infants (3).

Detection of genotypic variants leading to antimicrobial resistance. HRM has been applied to detect variants and specific SNPs in genes associated with antimicrobial resistance. It is of particular utility where phenotypic assays for antimicrobial resistance are difficult to perform or time and resource intensive.

Given the burden of tuberculosis (TB), in particular, drug-resistant TB, in regions of the developing world, reasonably inexpensive but rapid assays to detect drug resistance are required. A significant hurdle to timely use of resistance testing is the several weeks required for phenotypic testing. Multiple drugs are used to treat TB, each with specific mutation pathways leading to resistance. Thus, multiple genes targeted for mutation detection include *rpoB* for rifampin, *gyrA* for quinolones, and *katG* and the *mabA-inhA* promoter region for isoniazid. A recent study that targeted these regions for interrogation by HRM found ranges of sensitivity and specificity of 94% to 100% and 98% to 100% to detect resistance compared to phenotypic susceptibility testing (2). The false negatives that occurred were due to resistance mutations occurring outside the targeted regions, and the false positives were due to the detection of synonymous mutations. Nonetheless, the low cost and rapidity of this assay make it an attractive means of screening for gene mutations associated with drug resistance.

With the widespread use of oseltamivir for treatment of pandemic H1N1 influenza A 2009, there developed a need for molecular assays to rapidly detect mutations associated with oseltamivir resistance. Phenotypic assays such as neuraminidase inhibition assays are time-consuming and not widely used outside reference and research laboratories. Analysis using the HRM platform was one of several molecular techniques applied for detection of the oseltamivir resistance-conferring SNP at nucleotide position 823 of the neuraminidase gene that results in a histidine-to-tyrosine substitution at amino acid position 275. Due to the high mutational rate of the single-stranded RNA influenza virus, there are sequence variations in the regions flanking codon 275. Different strategies have been employed to prevent these variants from confounding HRM assays. These have included a single-base PCR amplification of position 823 followed by HRM analysis of the resulting amplicon (21) and a modified hybridization probe-based approach that utilizes an initial forward primer up to position 822 (25). Both approaches rely on primer sequence incorporation into the PCR product that results in the masking of variations in close proximity to the mutation of interest.

HRM has been applied to the detection of drug resistance mutations for *Plasmodium falciparum*. Daniels et al. have recently published methods to detect multiple mutations in six genes associated with *P. falciparum* drug resistance (4). Those investigators made use of an asymmetric PCR protocol to generate an excess of single-stranded DNA that was then bound by unlabeled 3′ blocked probes. These short (<50-bp) blocked probes covered the SNP(s) of interest and resulted in enhanced T_m separation between alleles compared to whole-amplicon melting analysis. Indeed, A-to-T mutations, which are the most challenging for HRM to distinguish, were convincingly discriminated. Furthermore, the assay detected different alleles in mixed parasite populations as well as separate copies of the *pfmdr1* locus in a single cultured parasite.

Detection of human genetic variants of importance to susceptibility to or treatment of infectious diseases. Detection of human genetic variants that either confer susceptibility to infectious diseases or are important for host response to treatment is likely to be a growing application of HRM analysis. An excellent example is the association with responses to interferon-based therapies for treatment of chronic hepatitis C virus infections of the rs126979860 polymorphism in the interleukin 28B gene. HRM analysis of a 59-bp region surrounding rs126979860 clearly dis-

criminated the CC, TT, and CT variants, with 100% correlation to sequencing results in 60 clinical samples (10).

LIMITATIONS OF HRM TECHNOLOGY AND APPLICATIONS

Probably the most important limitation of HRM analysis is that it may not reveal all the sequence variations in a DNA fragment. There is still a strong empirical component to the determination of which base changes are detected by HRM. Base differences that do not change the %G+C content are inherently more difficult to detect than those that do, and the probability that a base change is detectable by HRM is inversely correlated with DNA fragment length. In our experience and that of others, detecting single base changes in sequences of >200 bp is frequently problematic. The use of 3′ blocked probes to decrease the effective size of the fragment subjected to HRM analysis and so facilitate SNP interrogation is a promising methodological advance in efforts to overcome these limitations (4).

The inherent simplicity of HRM analyses would suggest that HRM analyses should be highly reproducible. However, reproducibility can be poor if there are variations in initial nucleic acid template or amplimer concentrations post-PCR (20, 21). The basis of this problem is under investigation in our research group, with initial indications being that the amplimer concentration affects the DNA-dye stoichiometry, which in turn impacts the melting characteristics (unpublished data). Multiplexed PCR and subsequent HRM analyses are difficult partly as a result of this sensitivity to variations in amplimer concentrations but also as a result of the need for the T_ms of the amplicons to be sufficiently separated to allow analysis at the HRM stage.

CONCLUSION

As understanding and awareness of HRM technology improve, we predict that HRM will find increasing applications in diagnostic and research laboratories. The low cost and use of generic instrumentation will likely provide a niche for HRM analysis, particularly for its utility in regions and laboratories with lesser resources but also as a complement to sequencing technologies.

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