Ann Ig 2017; 29: 293-316 doi:10.7416/ai.2017.2153

High Resolution Melting as a rapid, reliable, accurate and cost-effective emerging tool for genotyping pathogenic bacteria and enhancing molecular epidemiological surveillance: a comprehensive review of the literature

M. Tamburro[1], G. Ripabelli[1]

Key words: High Resolution Melting, infectious diseases, bacterial pathogens, genotyping, epidemiological surveillance
Parole chiave: High Resolution Melting, malattie infettive, batteri patogeni, genotipizzazione, sorveglianza epidemiologica

Abstract

Introduction. *Rapid, reliable and accurate molecular typing methods are essential for outbreaks detection and infectious diseases control, for monitoring the evolution and dynamics of microbial populations, and for effective epidemiological surveillance. The introduction of a novel method based on the analysis of melting temperature of amplified products, known as High Resolution Melting (HRM) since 2002, has found applications in epidemiological studies, either for identification of bacterial species or molecular typing, as well as an extensive and increasing use in many research fields. HRM method is based on the use of saturating third generation dyes, advanced real-time PCR platforms, and bioinformatics tools.*
Objective. *To describe, by a comphrehensive review of the literature, the use, application and usefulness of HRM for the genotyping of bacterial pathogens in the context of epidemiological surveillance and public health.*
Materials and Methods. *A literature search was carried out during July-August 2016, by consulting the biomedical databases PubMed/Medline, Scopus, EMBASE, and ISI Web of Science without limits. The search strategy was performed according to the following keywords: high resolution melting analysis and bacteria and genotyping or molecular typing. All the articles evaluating the application of HRM for bacterial pathogen genotyping were selected and reviewed, taking into account the objective of each study, the rationale explaining the use of this technology, and the main results obtained in comparison with gold standards and/or alternative methods, when available.*
Results. *HRM method was extensively used for molecular typing of both Gram-positive and Gram-negative bacterial pathogens, representing a versatile genetic tool: a) to evaluate genetic diversity and subtype at species/subspecies level, based also on allele discrimination/identification and mutation screening; b) to recognize phylogenetic groupings (lineage, sublineage, subgroups); c) to identify antimicrobial resistance; d) to detect and screen for mutations related to drug-resistance; e) to discriminate gene isoforms. HRM method showed, in almost all instances, excellent typeability and discriminatory power, with high concordance of typing results obtained with gold standards or comparable methods. Conversely, for the evaluation of genetic determinants associated to antibiotic-resistance or for screening of associated mutations in key gene*

1) Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, Campobasso, Italy

fragments, the sensitivity and specificity was not optimal, because the targeted amplicons did not encompass all the crucial mutations.
Conclusions. *Despite the recent introduction of sequencing-based methods, the HRM method deserves consideration in research fields of infectious diseases, being characterized by low cost, rapidity, flexibility and versatility. However, there are some limitations to HRM assays development, which should be carefully considered. The most common application of HRM for bacterial typing is related to Single Nucleotide Polymorphism (SNP)-based genotyping with the analysis of gene fragments within the multilocus sequence typing (MLST) loci, following an approach termed mini-MLST or Minim typing. Although the resolving power is not totally correspondent to MLST, the Simpson's Index of Diversity provided by HRM method typically >0.95. Furthermore, the cost of this approach is less than MLST, enabling low cost surveillance and rapid response for outbreak control. Hence, the potential of HRM technology can strongly facilitate routine research and diagnostics in the epidemiological studies, as well as advance and streamline the genetic characterization of bacterial pathogens.*

Introduction

High Resolution Melting (HRM) was first described in 2002, and has increasingly been used as a research tool (1). HRM was proven to be a highly sensitive and specific procedure for detecting genetic variation in PCR amplicons, and was widely and successfully employed in many clinical, genetic, and forensic applications, such as epidemiological surveillance studies, species identification and genotyping, identification of antibiotic resistance-associated determinants, as well as interrogation of Single Nucleotide Polymorphism (SNPs), alleles discrimination, and determination of DNA methylation status (2-10). In addition to SNPs discrimination, the HRM technique can be also used for detection of previously unrecognised polymorphisms. Any amplicon containing one or more base variations is likely to generate a different HRM profile compared to a control, although SNPs involving C-to-G or A-to-T substitutions can be difficult to distinguish. As a result, any sample with changes to curve shapes requires further analysis by sequencing, to confirm and identify the novel mutation(s) (11).

Melting separation of double stranded DNA (dsDNA) is a fundamental property of DNA that is monitored with changes in fluorescence intensity, and generates a characteristic sigmoidal curve. The melting temperature (Tm), correlated with sequence length and percent GC content, can be calculated by determining the peak of the derivative melting curve. Conventional melting is performed after PCR by any real-time instrument to verify both the amplified products purity and the sequence using hybridization probes. Through HRM, by raising temperature in post-amplification phase, the dsDNA separation profile is recorded in the presence of a saturating third generation fluorescent dye (i.e., LCGreen, SYTO9, EVAGreen), which produces an amplicon-specific melting profile, generally known as "Melt Type" (MelT) (12, 13). The advances in DNA melting techniques also include the development of instrumentation (i.e., Idaho Technology, Corbett Life Science, Roche Molecular Systems, Applied Biosystems, Qiagen, Thermo Fisher Scientific, Bio-Rad) characterized by highly controlled temperature transitions and data acquisition (14), allowing a precise assessment of sequence variations based on melting analysis, decreasing the burden of sequencing. SNPs can be identified without probes, and complex regions typed with unlabeled hybridization probes. For data

analysis, the use of the melting curve shape is also important, because sequence regions with different GC content may result in bi- or multiphasic melting curves, allowing allele discrimination (8).

By targeting ribosomal genes or highly conserved genes, HRM has been widely applied for the identification of microorganisms at the species level.

Although whole-genome sequencing (WGS) is likely to replace several molecular methods for microbial genotyping, the rapidity and simplicity of HRM-based assays (15), as well as the generic technology platform used, suggest that research protocols incorporating HRM as for first screening may have considerable value and effectiveness, especially if based on hypervariable gene fragments. Single-locus HRM analysis has been widely applied to bacteria and virus genotyping (16, 17), as well as multilocus HRM-based assays (9).

Objective

To describe and elucidate, through a comprehensive review of the literature, the applications and the use of the HRM as a powerful technique for the study of molecular epidemiology and surveillance of human bacterial pathogens.

Methods

The literature search was carried out between July and August 2016, by consulting biomedical databases PubMed/Medline, Scopus, EMBASE, and ISI Web of Science without limits. The search strategy was performed according to the following keywords: high resolution melting analysis and bacteria and genotyping or molecular typing.

The inclusion criteria considered for documents eligibility were pertinence and relevance according to the aims of this review. Thus, the articles evaluating the application of HRM for bacterial pathogens genotyping were selected and reviewed, taking into account the objective of each study, the rationale explaining the use of HRM, and the main results obtained, also in comparison with gold standards and/or alternative methods, when available.

Database search results

The main outcomes of database interrogation were related to specific pathogens genotyped according to the HRM method, which was used for molecular typing of both Gram-positive (Table 1), and Gram-negative bacteria (Table 2). The main findings are illustrated within two separate paragraphs, Gram-positive and Gram-negative pathogens.

HRM typing of Gram-positive bacteria

Bacillus anthracis

B. anthracis is the etiologic agent of anthrax, a zoonotic disease mainly associated with wild herbivores and domestic animals. The bacterium can infect humans by cutaneous, gastrointestinal or respiratory routes (18), and spreads through close occupational proximity to infected livestock by handling infected animals. Molecular genotyping of *B. anthracis* is important for identifying strains from different geographic areas, and tracing those released deliberately for bioterrorism. The method universally adopted for this purpose is Multiple-Locus Variable-number tandem repeat Analysis (MLVA) that differentiates allelic variants, based on the length polymorphisms with the amplification of multiple chromosomal loci carrying Variable Number Tandem Repeats (VNTRs) and sizing the fragments (19). Fortini and co-workers (20) examined the feasibility of HRM analysis to differentiate VNTR allelic variants by amplifying 25 VNTR loci in 12 strains from the Istituto

Table 1 - Evidence-based reviewed studies using HRM for genotyping Gram-positive pathogens

Gram-positive bacteria	Objective	Reference
B. anthracis	To screen14 phylogenetically informative SNPs that subtype the species into 13 major sublineages or subgroups	(5)
	To genotype a set of 13 phylogenetically informative SNPs into one of the 12 major sublineages or subgroups	(11)
	To screen the allelic variants for 6 selected loci	(20)
C. difficile	To identify ribotypes 027/078 strains	(27)
	To investigate among strains with known PCR-ribotypes for the respective SNPs within *rpo*B region for discriminating mutant and wild-type DNA	(26)
Enterococcus spp.	To detect linezolid-resistant based on the polymorphism G2576T in the *23S rRNA* gene mutation	(30)
E. faecium	To genotype by interrogating eight SNPs derived from the *E. faecium* MLST database	(29)
Group A streptococci (GAS)	To genotype isolates based on the optimized SNPs	(70)
	To identify the seven *emm* types 3, 4, 6, 12, 28, and 89, together with *emm*11 for the rapid typing of strains	(71)
	To detect and differentiate the macrolide resistance genes *mefA*, *ermTR* and *ermB*	(95)
L. monocytogenes	To genotype strains based on a specific locus of the internalin B gene	(36)
	To genotype strains through the discrimination of DNA sequences variations within *inl*B and *ssr*A genes	(37)
MRSA	To facilitate allele discrimination analyzing eight SNPs derived from the *S. aureus* MLST database	(8)
	To identify ST93 and reveal diversity within the *spa* locus of this lineage	(17)
	To genotype isolates based on the *spa* polymorphic region X	(43)
	To genotype isolates by spa-typing *spa*-typing	(45)
	To genotype isolates by spa-typing *spa*-typing	(46)
	To implement a novel approach for epidemiological surveillance focusing on SNPs within *yqiL* gene	(47)
	To simultaneously detect the presence of the Panton-Valentine leukocidin gene and discriminate histidine and arginine isoforms	(48)
	To determine the prevalence of the healthcare-associated clones ST22 and ST239	(49)
	To genotype isolates by *spa*-typing	(55)
M. tuberculosis	To detect rifampin and isoniazid resistance targeting *rpo*B, *rpo*B-516, *kat*G, and *inh*A	(7)
	To scan for mutations in the *rpo*B gene associated to rifampicin resistance	(52)
	To detect mutations conferring resistance to rifampin and isoniazid targeting *rpo*B and regions of *kat*G and *inh*A promoter	(53)
	To scan for mutations in the *rpo*B, *inh*A, *ahp*C, and *kat*G genes and/or promoter regions for the detection of rifampin and isoniazid resistance	(54)
	To detect isoniazid, rifampin, and ofloxacin resistance by targeting resistance-associated mutations in *kat*G, *mab*A-*inh*A promoter, *rpo*B, and *gyr*A genes	(55)
	To detect mutations in drug resistance-associated *rpo*B, *kat*G and *rps*L genes in isolates phenotypically resistant to rifampicin, isoniazid and streptomycin	(56)
	To detect mutations within *gyrA*, *rpsL*, and *rrs* for the determination of fluoroquinolone and streptomycin resistance	(57)
	To detect resistance to rifampicin and isoniazid by scanning for mutations in *rpo*B and *kat*G genes	(58)
	To rapidly detect pyrazinamide resistance	(59)
	To detect the mutations responsible for the resistance of rifampicin, isoniazid, ethambutol, and streptomycin	(60)
	To rapidly detect resistance conferring mutations in *rpo*B and *kat*G genes	(61)
	To detect pyrazinamide resistance targeting *pnc*A gene	(62)
	To detect rifampicin resistance	(63)
S. epidermidis	To identify the G2576T point mutation in domain V of the *23S rRNA* genes attributed to linezolid resistance	(66)

Superiore di Sanità (Rome, Italy). The use of HRM provided reproducible intra-experimental melting curves for the same alleles, and discriminated alleles with different numbers of repeat units. The Authors also selected 6 out of 25 loci to easily differentiate melting curves and distinguish the strains. Although cluster analysis of these loci characterized 7 genotypes compared to 10 genotypes identified with MLVA, this application underlined the use of HRM for rapid screening of *B. anthracis* VNTR loci.

The analysis of canonical SNPs is a fast way to determine the clonal sublineages of *B. anthracis*. Derzelle and co-workers (11) developed a HRM-based method to genotype a set of 13 useful SNPs within *B. anthracis* genome. The assays for SNPs discrimination were applied to 100 isolates collected in France from 1953 to 2009, which were discriminated into one of the 12 major sublineages or subgroups. In a recent study (21), a HRM method based on the screening of 14 SNPs within *B. anthracis* genome allowed the identification of 13 major sublineages or subgroups.

Clostridium difficile

C. difficile infection is increasingly recognized as an emerging healthcare problem worldwide (22). In the past decades, the incidence of infection has raised due to an improved disease detection, a significant increase of the vulnerable population, including the elderly and immunocompromised individuals (23), and the circulation of hypervirulent strains and a growing prevalence of asymptomatic *C. difficile* carriage (24). Recent typing strategies, such as WGS and MLVA, used for tracking transmission across healthcare facilities and countries, have demonstrated a wide heterogeneity of strain types, which reflected different stages of epidemic spread (25). Rifaximin constitutes a promising treatment of recurring infections, but the constant exposure has led to the development of resistance due to point mutations in the β-subunit of the RNA polymerase (*rpoB*) gene (25).

In a study (26), 348 *C. difficile* strains with known PCR-ribotypes were analyzed through HRM for SNPs detection within *rpoB* region, by discriminating the melting behavior of mutants to wild-type DNA strains. Sequencing analysis of a specific fragment identified 16 different *rpoB* sequence types (STs), and displayed 24 different SNPs. Fifteen of these SNPs were located within the *rpoB* region targeted with HRM, resulting in 11 different melting curve profiles demonstrating the HRM analysis as a fast and cost-effective method for the identification of rifaximin resistant strains, and for SNPs subtyping.

de Almeida Monteiro and co-workers (27) used multiplex-PCR followed by HRM to identify *C. difficile* ribotypes 027/078 strains. Among 116 patients included in the study, 11 were *C. difficile* positive, and were evaluated for the presence of hypervirulent characteristics. Among these, 10 clinical strains were non-toxigenic. Compared to controls, none of the isolates had toxin gene profiles similar to ribotypes 027/078, and these findings were confirmed by HRM, because no isolates showed peaks compatible with PCR ribotypes 027/078.

Enterococcus faecium

Enterococci are commensal of the gastrointestinal tract of humans, mammals, and insects, but have also become globally significant nosocomial pathogens, and *E. faecium* and *E. faecalis* are the most clinically relevant species. *E. faecium* infections are commonly associated with hospitalized patients and rarely encountered in community settings (28). Hospital-associated clones are characterized by the acquisition of adaptive genetic elements, including genes involved in metabolism, biofilm formation, and antibiotic resistance.

Table 2 - Evidence-based reviewed studies using HRM for genotyping Gram-negative pathogens

Gram-negative bacteria	Objective	Reference
B. pertussis	To rapid detect erythromycin-resistant strains based on the A2047G mutation in *23S rRNA* genes	(73)
C. coli	To genotype strains targeting the clustered regularly interspaced short palindromic repeat (CRISPR) lous	(78)
C. jejuni	To identify known MLST alleles	(77)
C. trachomatis	To genotype based on the target of variable segment 2	(80)
Enterobacteriaceae, *P. aeruginosa, A. baumannii*	To detect the presence of *bla*KPC and differentiate between KPC-2-like and KPC-3-like alleles	(85)
Escherichia coli O157	To study epidemiologically unlinked strains and differentiate into clades	(82)
K. pneumoniae	To genotype strains based on six SNPs derived from concatenated MLST sequences	(84)
	To detect plasmid-mediated AmpC genes	(87)
Leptospira	To identify genotypes at serovar level	(89)
P. aeruginosa	To genotype a collection of environmental and clinical strains	(91)
	To genotype strains utilizing ten SNPs	(92)
Salmonella enterica	To detect *gyr*A mutations that cause quinolone resistance in typhoid and paratyphoid fever *Salmonella* spp.	(95)
	To subtype at serotype-specific level *Salmonella* based on SNPs in fragments of *flj*B, *gyr*B, and *ycf*Q	(96)
	To rapidly screen for mutations in quinolone-resistant determining region of gyrase and topoisomerase IV genes	(97)
	To differentiate between the allelic variants in 5 tandem repeat loci	(98)
S. sonnei	To define phylogeny and identify lineages/sublineages by typing 6 informative SNPs	(100)
	To identify and genotype the emerging multidrug-resistant clones based on selected SNPs	(101)
V. cholerae	To genotype strains	(103)
Y. enterocolitica	To determine SNPs and the genetic diversity of strains	(106)
Y. pestis	To genotype isolates analyzing the polymorphic DNA regions	(107)
Y. pseudotuberculosis	To genotype isolates based on differences in 10 SNPs in the *16S rRNA*, *gln*A, *gyr*B and *rec*A sequences	(10)

Tong and co-workers (29) developed SNP-based HRM technique for genotyping 85 *E. faecium* isolates. Eight informative SNPs were derived from *E. faecium* MLST database, and the amplified fragments containing them were analyzed by HRM. Results were compared with those obtained with MLST, Pulsed-field gel electrophoresis (PFGE) and allele specific real-time PCR. *In silico* analysis on predicted curves of each fragment for all 567 STs in the MLST database together with HRM data resolved *E. faecium* isolates into 231 MelTs and provided a Simpson's Index of Diversity of 0.99, while allele specific real-time PCR scheme resolved 61 SNP types with a Simpson's Index of Diversity of 0.95. Amongst the isolates, there were 13 PFGE patterns, 17 STs, 14 MelTs and eight SNP types, proving a concordance between the methods. Moreover, Alonso and co-workers (30) designed a HRM procedure to detect linezolid-resistant *Enterococcus* spp. based on G2576T polymorphism, most frequently associated with resistance. Between 2009 and 2012, 23 *E. faecium* and 2 *E. faecalis*

linezolid-resistant isolates were recovered. The G2576T mutation was identified in all isolates using *Nhe*I PCR-RFLP, and confirmed by DNA sequencing. HRM analysis detected G2576T mutation, and correctly discriminated between susceptible and resistant enterococcal isolates.

Listeria monocytogenes

L. monocytogenes is the etiologic agent of listeriosis, a severe infection especially for specific risk population groups such as the elderly, immunocompromised individuals and pregnant women (31). Human listeriosis is generally acquired through contaminated food. Despite the low incidence, the case-fatality rate of listeriosis is high, indicating a great burden of disease for public health (32, 33). Several molecular protocols revealed that *L. monocytogenes* comprises phylogenetic groups (PG), also correlated with serotypes (34, 35): PG I.1 with serotype 1/2a, 3a; I.2 with 1/2c, 3c; II.1 with 4b, 4d, 4e; II.2 with 1/2b, 3b, 7; III with 4a, 4c. For outbreak detection and investigation, the discriminatory power of the classical multiplex-PCR based serotyping methods is not sufficient, and the use of PFGE is necessary, representing the current gold standard for *L. monocytogenes* typing although it is time-consuming, hampers inter-laboratory exchange and comparison of results (15).

Pietzka and co-workers (36) proposed a HRM assay based on internalin B (invasion protein, encoded by *inl*B gene) fragment gene for *L. monocytogenes* typing, and melting curve profiles were confirmed by sequence analysis. The 192 *L. monocytogenes* isolates tested yielded 15 specific HRM profiles, which corresponded to 18 distinct STs. HRM profiles correlated with phylogenetic groups, and allowed differentiation of 1/2a, 1/2b, and 4b isolates, responsible for more than 96% of human listeriosis cases, proving to be accurate and less expensive compared to classical serotyping or protocols for phylogenetic group classification.

HRM was also applied for genotyping 55 *L. monocytogenes* isolates, by discriminating DNA sequences variations of *inl*B and *ssr*A (coding for tmRNA with functions of both tRNA and mRNA) genes in two different assays (37). A dendrogram produced by Random Amplified Polymorphic DNA (RAPD) analysis for the same isolates was used for comparison. HRM showed a higher resolution to differentiate *L. monocytogenes* isolates, indicating this method as a valid alternative to RAPD analysis.

Methicillin-resistant Staphylococcus aureus (MRSA)

S. aureus is one of the most serious human pathogens, responsible for a wide spectrum of infections, mainly associated to methicillin-resistant *S. aureus* (MRSA) (38), representing the major causative agent in wound nosocomial infections (39). Staphylococcal protein A (encoded by *spa* gene) is an important virulence factor enabling the bacterium to evade host immune responses (40). Typing based on the highly variable region of *spa* gene is one of the most common methods, yielding genotypes known as "*spa*-types" that contribute to understand diversity, carriage, and transmission of strains in different populations (41), and also to facilitate communication and data comparison between national and international laboratories. Moreover, SCC*mec* (*S*taphylococcus *C*assette *C*hromosomal *mec*), is a mobile genetic element carrying the key determinant for broad-spectrum β-lactam resistance encoded by *mec*A gene. The emergence of MRSA is due to the acquisition and insertion of SCC*mec* element into the chromosome of susceptible strains. The SCC*mec* elements differ substantially in their structural organization and genetic content, and can be classified into types and subtypes. MRSA clones are defined by combined SCC*mec* type and MLST chromosomal background (42).

Stephens and co-workers (43) described the use of HRM to analyze 22 known *spa* sequences of 44 MRSA isolates. The assay generated twenty different profiles, and only two types were unresolved, which differed by only 1 bp (base pairs) sequence length. Tong and co-workers (17) developed HRM analysis to detect ST93, a singleton clone unique to Australia, and to reveal diversity within *spa* locus. The results supported early acquisition or multiple independent acquisitions of SCC*mec* by ST93 methicillin-susceptible *S. aureus* (MSSA), and the coexistence of MSSA and MRSA versions of the same lineage. Furthermore, Chen and co-workers (44) analyzed 55 clinical MRSA isolates based on *spa* typing through conventional sequencing and HRM method. Twelve different *spa*-types were observed by sequencing, and all *spa* genotypes were differentiated by divergent melting curves and plot analysis, hence, demonstrating 100% concordance and the usefulness of HRM approach for monitoring MRSA community transmission and for nosocomial outbreak control. Mazi and co-workers (45) evaluated HRM as a *spa*-typing method on 50 MRSA isolates. Nineteen *spa*-types were identified by sequencing, of which fifteen had a distinct Tm and were unambiguously genotyped. The remaining four *spa*-types were not separated through Tm analysis, sharing the same Tm; however, these *spa*-types were successfully separated with melting shapes analysis. A similar HRM *spa*-typing of MRSA isolates was evaluated by Mayerhofer and co-workers (46), and results indicated that HRM could be a fast and accurate screening tool to detect the most frequent endemic *spa*-types, and to exclude the non-endemic ones within a hospital.

In another study (47), a HRM scheme was implemented focusing on specific SNPs within the *yqi*L (encoding Acetyl coenzyme A acetyltransferase) housekeeping gene. HRM data were compared with those obtained by MLST, showing 94% agreement. Seven out of eleven MelTs corresponded to the correct STs with 100% sensitivity and specificity, and good accuracy values were obtained for the remaining. The method allowed discrimination of the major MRSA allelic variants, and showed advantages in terms of cost reduction and time requirements as compared to MLST.

A HRM assay was designed to simultaneously detect the Panton-Valentine leukocidin gene (encoding for synergohymenotropic toxins) and discriminate histidine and arginine isoforms among 223 *S. aureus* isolates from northern Australia (48). The results showed that isoforms clustered by clonal complex (CC), and all CC93 isolates harbored the arginine isoform.

Lilliebridge and co-workers (8) evaluated a MLST-based HRM method for SNPs detection, to facilitate allele discrimination. HRM analysis on 94 *S. aureus* isolates of known ST or CC resolved 268 MelTs, providing a Simpson's Index of Diversity of 0.978. These results further showed high concordance with CCs as defined by MLST. Recently, Jeremiah and co-workers (49) determined the prevalence of the healthcare-associated ST22 and ST239 clones using a MLST-based HRM scheme. The results confirmed that isolates typed as CC22 or CC239 were consistent with known ST22 and ST239.

Mycobacterium tuberculosis

Tuberculosis remains a leading cause of morbidity and mortality accounting for 1.5 million deaths and 9 million infections worldwide (50). The prolonged duration of treatment with multiple drugs can result in patient non-compliance, unsuccessful cure and frequent selection for multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (51). Several studies investigated the use of HRM to screen for mutations related to drug resistance in *M. tuberculosis*. In the study of Pietzka et al.

(52), HRM was used to scan for mutations in *rpoB* gene among 49 MDR strains and 19 susceptible isolates, as previously determined by BACTEC-MGIT960 System drug susceptibility testing (Becton, Dickinson and Company). The approach allowed the correct identification of 44 MDR isolates and all non-MDR isolates, although three of five MDR isolates were falsely identified as non-resistant. The combined HRM analysis of all isolates showed 95.9% sensitivity and 100% specificity, 100% and 99.9% positive and negative predictive values, indicating that it is a valuable screening method, with minimal requirements of cost and time.

Choi and co-workers (7) evaluated HRM as a tool for detecting rifampin (RIF) and isoniazid (INH) resistance in 217 *M. tuberculosis* clinical isolates of known resistance phenotype, by using four pairs of primers for *rpo*B, *rpo*B-516, *kat*G (catalase-peroxidase), and *inh*A (NADH-dependent enoyl reductase) genes, and results were confirmed by sequencing. All but one of the 73 RIF-resistant (R) strains and all 124 RIF-susceptible (S) isolates were correctly identified. Ninety INH-R strains harboring *katG* or *inhA* mutations, or both, and all INH-S strains were discriminated. Ten phenotypically INH-R strains not harboring *katG* or *inhA* mutations were not detected. HRM was proved to be a useful and accurate method for detection of RIF and INH resistance.

Ramirez and co-workers (53) described a HRM assay to identify *M. tuberculosis* strains harboring mutations at some loci, as RIF resistance determinant region of *rpo*B, and regions of *katG* and *inhA* for RIF and INH resistance detection, respectively. The assay demonstrated sensitivity and specificity of 91% and 98% for the detection of RIF resistance, 87% and 100% for INH, 85% and 98% for MDR strains detection. Ong and co-workers (54) developed a HRM assay to scan for mutations in *rpoB*, *inhA*, *ahpC alkyl* (hydroperoxide reductase subunit), and *katG* genes. For the assay, 23 drug-resistant isolates and 40 susceptible isolates were tested. All sequence mutations within targeted regions were correctly identified, showing 100% sensitivity and specificity. For blinded samples, the specificities and sensitivities were 89.3% and 100% for RIF resistance, 98.1% and 83.3% for INH resistance, indicating that HRM can be used for mutation scanning and for detecting drug-resistant *M. tuberculosis*.

In the study of Chen and co-workers (55), HRM assay was developed to detect isoniazid, rifampin, and ofloxacin resistance in 17 drug-resistant and 11 susceptible *M. tuberculosis* strains, targeting resistance-associated mutations in *katG*, *mabA-inhA* promoter, *rpoB*, and *gyrA* (DNA gyrase subunit A) genes. HRM results matched with 18 mutations previously identified by sequenced drug-resistant isolates, and no mutations were found in susceptible isolates. Among 87 additional isolates with known resistance phenotypes, HRM identified *katG* and/or *mabA-inhA* mutations in 66 of 69 isoniazid-resistant isolates, *rpoB* mutations in 94.4% rifampin-resistant isolates, and *gyrA* mutations in all 41 ofloxacin-resistant isolates. Yadav and co-workers (56) detected rifampicin, isoniazid and streptomycin resistance in phenotypically resistant clinical isolates, analyzing by HRM mutations in *rpoB*, *katG* and *rpsL* (30S ribosomal protein S12) genes. HRM generated 11 distinguishable melt curves representing eight mutation types. For the three drugs, respectively, the sensitivity of HRM assay was 93.1%, 80% and 61.8% compared to the phenotypic resistance patterns, and 93.1%, 93.3% and 100% respect to sequencing.

Lee and co-workers (57) used HRM to determine fluoroquinolone and streptomycin resistance in 92 *M. tuberculosis* isolates, detecting mutations within *gyr*A, *rps*L, and *rrs* (16S ribosomal RNA), and results were verified by sequencing. Compared with drug susceptibility testing, HRM sensitivity

and specificity were 74.1% and 100% for fluoroquinolone resistance detection, 87.5% and 100% for streptomycin. Five isolates with low-resistance to ofloxacin had no detectable mutations in *gyr*A, and one fluoroquinolone-resistant isolate had a mutation in a region not encompassed by the assay. Six streptomycin-resistant strains had undetectable mutations by both HRM and sequencing. The HRM assay was proven to be able to determine fluoroquinolone and streptomycin resistance, and facilitated selection of treatment options. Nour and co-workers (58) developed a HRM assay to simultaneously detect resistance to RIF and INH among isolates of known phenotypes. HRM curves were generated for each isolate to scan for mutations in the *rpo*B and *kat*G genes to detect RIF and INH resistance, respectively. The Resazurin microtiter assay (REMA) plate, a method for detecting MDR by using an oxidation-reduction indicator, was evaluated on the same isolates, and results of both techniques were compared to the gold standard of proportion method. Sensitivity, specificity, positive and negative predictive values, and accuracy of REMA assay for RIF and INH testing were 100%. HRM results for RIF susceptibility were 92.3%, 100%, 100%, 94.4%, and 96.7%, respectively, and 85%, 100%, 100%, 76.9%, and 90% for INH, respectively. The use of HRM was also evaluated for the detection of pyrazinamide resistance (59) on 48 *M. tuberculosis* strains. Using BACTEC-MGIT960 for pyrazinamide susceptibility testing, 62 isolates were found resistant and 65 susceptible. Through HRM, 53 strains were resistant and 64 susceptible, with sensitivity and specificity of 85.5% and 98.5%, respectively, proving a good correlation with BACTEC-MGIT960.

Nagai and co-workers (60) developed and evaluated a HRM assay to detect the most frequent mutations responsible for the resistance of the anti-tuberculosis drugs, rifampicin, isoniazid, ethambutol and streptomycin. HRM was used to reveal dominant mutations in *rpoB*, *katG*, *mab-inhA*, *embB* (arabinosyltransferase B), *rpsL* and *rrs* genes. By analyzing the melting shapes in 40 *M. tuberculosis* isolates, the method discriminated between mutant and wild-type, and results were completely consistent with sequencing. Haeili and co-workers (61) evaluated the HRM for rapid detection of resistance targeting mutations in *rpo*B and *kat*G genes. The analysis allowed correct identification of 19 of the 20 phenotypically RIF-resistant and all RIF-susceptible isolates. All INH-susceptible isolates generated wild-type curves, and 18 out of 21 INH-resistant isolates exhibited mutant type curves. However, one RIF-resistant and three INH-resistant isolates were falsely identified as susceptible, confirmed to have no mutation by sequencing. HRM assay was developed to detect pyrazinamide (PZA) resistance (62). Mutations were detected in all PZA resistant samples, whereas all susceptible strains had no mutation in the *pncA* (pyrazinamidase/nicotinamidase) gene. Results were concordant with BACTEC-MGIT960 susceptibility testing, and detected mutation were consistent with sequencing. Malhotra and co-workers (63) evaluated sensitivity and specificity of a line-probe assay (LPA) and HRM analysis in comparison with BACTEC-MGIT960 system for rifampicin resistance detection among 219 *M. tuberculosis* isolates. HRM enabled identifying 93/103 isolates resistant and 113/116 susceptible on BACTEC-MGIT960, with 90.3% and 97.4% sensitivity and specificity, respectively. HRM further identified 117/119 LPA-susceptible and 94/100 resistant isolates, with 98.3% specificity and 94% sensitivity, with a valid potential for drug resistance screening. Two susceptible isolates on LPA assay but resistant on HRM showed silent mutations by sequencing, while six isolates identified as susceptible by HRM but resistant on LPA showed particular mutations.

Staphylococcus epidermidis

S. epidermidis is part of the human epithelia microflora and has a benign relationship with the host (64), but has also emerged as pathogen that causes infections involving medical devices, such as peripheral or central intravenous catheters (65). Gabriel and co-workers (66) described a HRM-PCR assay for detection of linezolid resistance mediated by G2576T point mutation in the *23S rRNA* genes. The method demonstrated 100% correlation with the previously established *Nhe*I restriction assay.

Group A Streptococcus

Lancefield Group A Streptococci (GAS) or *S. pyogenes* (67) are associated with substantial worldwide morbidity and mortality with more than 34 million affected people, over 345,000 deaths and 10 million disability-adjusted life years lost per year (68). The infections are mostly associated with complications such as pharyngitis, impetigo, and non-suppurative immune syndromes. Deaths occur in children, adolescents, and young adults, particularly in pregnant women in low- and middle-income countries.

Slinger and co-workers (69) developed a HRM multiplex-PCR assay targeting genes encoding for macrolide resistance determinants, such as *mef*A (macrolide-efflux protein), ermTR and ermB (erythromycin resistance methylase) genes, while presence of a single *speB* (pyrogenic exotoxin B) peak indicated that these resistance genes were absent. HRM results were 100% concordant with agarose gel electrophoresis, showing the advantages of assay completion within <1 h.

S. pyogenes genotyping methods as *emm* (peptidoglycan-spanning domains) sequence typing are based on regions under considerable selective pressure, and MLST is not widely used due to high cost. A robust and cost-effective alternative was developed deriving 10 SNPs from MLST database (70). The fragments were predicted to generate three to six resolvable HRM curves. The combination of curves across each fragment were used to generate a MelT for each ST. The 525 STs in the *S. pyogenes* MLST database were predicted to resolve into 298 distinct MelTs with Simpson's Index of Diversity of 0.996. The method was validated on clinical isolates by examining 70 STs, and curves discriminated into 65 distinct MelTs. A multiplex-PCR and HRM analysis was also used to identify seven *emm* types 3, 4, 6, 12, 28, and 89, together with *emm*11, allowing a rapid typing of GAS strains (71).

HRM typing of Gram-negative bacteria

Bordetella pertussis

Pertussis is a severe respiratory infection caused by *B. pertussis*, with estimated 16 million cases and 195,000 deaths globally. Ongoing research using molecular tools can improve the understanding of epidemiology, pathogenesis, and immunology (72). Zhang and co-workers (73) used a HRM assay for rapid detection of erythromycin-resistant *B. pertussis* isolated from nasopharyngeal specimens of two asymptomatic schoolchildren. Sequencing analysis confirmed the homogeneous A2047G mutation in *23S rRNA* genes of the two isolates.

Campylobacter jejuni and Campylobacter coli

Over the last decade, the incidence and prevalence of campylobacteriosis have increased in both developed and developing countries (74), and disease spectrum includes acute enteritis, extra-intestinal infections, and post-infectious complications (75). The most common species associated with human illness is *C. jejuni*, but other species also causes infection (76). Lévesque and co-workers (77) evaluated HRM to identify known MLST alleles of 47 characterized *C.*

jejuni, including the most prevalent STs and alleles. The 92% of the alleles were resolved in 35 hours of laboratory time and the cost of reagents per isolate was $20 compared with $100 for sequencing, highlighting the usefulness of HRM to complement sequence methods for resolving SNPs.

Recently, 63 *C. coli* isolated from humans, animals, food and environment in Brazil were genotyped (78) to better understand genotypic diversity and compare the suitability of three different methods. In addition to PFGE and sequencing of *fla*A gene short variable region (*fla*A-SVR), HRM of the CRISPR (clustered regularly interspaced short palindromic repeat) was performed. The analysis detected 22 different *fla*A-SVR alleles, and seven new alleles. Some clinical and non-clinical *C. coli* isolates had ≥80% similarity by both PFGE and *fla*A-SVR sequencing, while CRISPR locus-based HRM allowed to identify four different melting profiles. The Simpson's Index of Diversity for PFGE, *fla*A-SVR sequencing and CRISPR-HRM was 0.986, 0.916 and 0.550, respectively, indicating that targeting CRISPR locus by HRM is not suitable for *C. coli* subtyping.

Chlamydia trachomatis

Genital infections with *C. trachomatis* continue to be a major health problem worldwide. While clearance can occur in some individuals, others develop chronic infection or reinfections. In females, chronic asymptomatic infections are particularly common, and can lead to pelvic inflammatory disease and infertility (79).

A HRM for *C. trachomatis* genotyping was developed and applied to 11 sexually transmitted infection-related genotypes, D through K and L1 through L3 (80), by the identification of the variable segment 2 target. All genotypes were distinguished from each other. Particularly, genotypes F, G, H, I, J, K, L2 and L3 were directly differentiated, whereas D, E and L1 were discriminated by a second analysis with fewer curves or by heteroduplex formation with a known reference strain.

STEC Escherichia coli including O157

Shiga toxin-producing *Escherichia coli* (STEC) are responsible for gastrointestinal diseases reported in many outbreaks worldwide. Effective STEC detection, characterization and typing are critical for biomedical research laboratories (81).

Recently, strains of enterohemorrhagic *E. coli* O157 (O157) isolated from patients with hemorrhagic colitis (HC), patients with no HC and asymptomatic carriers were differentiated into clades using HRM (82). The majority of the strains were correctly clustered by minimum spanning tree analysis, and strains in the main clades showed linkage disequilibrium, confirming the clade differentiation. The number of O157 strains in the different clades isolated from HC patients and the non-HC group was significantly different, indicating that strains in diverse clades had dissimilar pathogenicity for hemorrhagic colitis.

Klebsiella pneumoniae

The emergence of carbapenem-resistant pathogens represents a serious threat to public health worldwide. Particularly, the increasing prevalence of carbapenem-resistant *K. pneumoniae* is a major source of concern, because characterized by multidrug resistance, involving penicillins, cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors (83). *K. pneumoniae* carbapenemases (KPCs), oxacillinase-48 (OXA-48) and New Delhi metallo-β-lactamase (NDM) type are widely disseminated (83), although the prevalence of resistant strains differs geographically. Because carbapenemase-producing *K. pneumoniae* are often resistant to most β-lactam antibiotics and non-β-lactam molecules, therapeutic options available to treat infection caused by these strains are

limited to colistin, polymyxin B, fosfomycin, tigecycline, and selected aminoglycosides.

Andersson and co-workers (84) applied SNPs-based genotyping using HRM analysis of fragments within MLST loci (called as Minim typing approach) for *K. pneumoniae*. Six SNPs were derived from concatenated MLST sequences, and specific nucleotides were designed. Minim typing using six fragments was predicted to provide a Simpson's Index of Diversity of 0.979. The HRM method was tested on *K. pneumoniae* and MLST was further performed. The resulted HRM alleles were in accordance with GC content, and the Minim typing identified known and new STs, and corresponded to current MLST data.

Roth and Hanson (85) developed a real-time PCR assay complemented with HRM to detect the bla_{KPC} and for genotyping KPC-2 and KPC-3-like alleles. A total of 166 clinical isolates of Enterobacteriaceae, including *K. pneumoniae*, were tested, of which 66 clinical isolates produced KPC β-lactamase. HRM demonstrated that 26 had KPC-2-like melting peak temperatures, while 40 had those corresponding to KPC-3-like. Sequencing of 21 amplified products confirmed the melting peak results, and identified 9 isolates carrying bla_{KPC-2} and 12 isolates with bla_{KPC-3} in less than 3 h.

AmpC β-lactamases are associated with resistance to broad-spectrum penicillins and cephalosporins, monobactams, and cephamycins, and the encoding chromosomal genes are grouped into six families, bla_{MOX}, bla_{FOX}, bla_{CMY-2}, bla_{DHA}, bla_{ACC}, and bla_{ACT} (86).

Geyer and Hanson (87) proposed a multiplex real-time PCR assay using HRM for AmpC genes detection in *E. coli*, *K. pneumoniae*, *K. oxytoca* and *Salmonella* spp. All isolates tested were compared to data obtained using the PCR gold standard for AmpC detection. The HRM sensitivity and specificity were both 100% based on PCR confirmation.

Leptospira

Leptospirosis is an endemic zoonosis in tropical areas (88), and *L. interrogans* is the principal causative species of human infections. In a recent study (89) combined HRM method with an approach using primers to amplify two VNTR for typing at species and subspecies level was carried out. Using three selected VNTR primers to genotype at the subspecies level, the probability of a strain belonging to a specific cluster was ≥ 0.95. HRM enabled the identification at the species and serovar level by detecting genotypes with high resolution power (Simpson's Index of Diversity of 0.984).

Pseudomonas aeruginosa

P. aeruginosa is one of the most frequent opportunistic pathogens responsible for nosocomial pneumonia (90), and effective and discriminatory typing systems are essential for epidemiological surveillance. Naze and co-workers (91) developed a MLVA typing coupled with HRM for genotyping environmental and clinical strains, and results were compared with amplified fragment length polymorphism (AFLP) analysis as a reference method. Twenty-five and 28 genotypes were identified respectively with both techniques; based on a reference index of discriminatory power of 1.00 for AFLP typing, the index of discriminatory power calculated for 61 strains was 0.99 for VNTR-HRM, demonstrating to be highly reproducible and discriminative. Anuj and co-workers (92) identified the presence of clonal *P. aeruginosa* isolates from patients with cystic fibrosis by using real-time PCR and HRM targeting selected SNPs from seven MLST housekeeping genes. The sequence data were investigated for SNPs enabling discrimination of the three major clonal strains and all other strains. Twenty candidate SNPs were initially chosen on 106 isolates and five reference strains by HRM analysis. Data were used to select a ten-SNP profile. HRM based on 20 SNPs had

the highest discriminatory power (0.941), while the assay on ten SNPs was equivalent to PFGE (0.921).

Salmonella enterica

Salmonella is a human pathogen found in both developed and developing countries, causing clinical diseases ranging from mild gastroenteritis to septicemia (93). Quinolones are usually employed in treating these infections. However, the worldwide emergence of resistant strains has raised serious concerns. Among *Salmonella* the major mechanisms for quinolone-resistance comprises altered protein targets for quinolones, decreased uptake of quinolones, and DNA gyrase protection via plasmid-derived *qnr* genes (94). DNA gyrase and topoisomerase IV are two enzymes involved in bacterial DNA replication. Quinolones bind to gyrase/topoisomerase IV-DNA complex and inhibit DNA replication. Mutations in genes encoding DNA gyrase and topoisomerase IV may confer resistance to quinolones, and the altered structures prevent the binding (94).

Slinger and co-workers (95) described a HRM assay to detect *gyr*A mutations causing quinolone resistance in *Salmonella* spp. responsible of typhoid and paratyphoid fever. The presence of *gyr*A mutations led to small but consistent changes in the melting temperature of the amplicons, allowing differentiation of resistant from susceptible isolates. A triplex gene-scanning assay based on HRM analysis was developed by Zeinzinger et al. (96) for serotype-specific subtyping of *Salmonella* isolates targeting SNPs in *flj*B (encoding phase 2 flagellin), *gyr*B (DNA gyrase subunit B), and *ycf*Q (transcriptional repressor) fragment genes. Scanning of *flj*B, *gyr*B, and *ycf*Q allowed the unequivocal identification of 37 serotypes. To differentiate the most frequent *Salmonella* serotype, an additional single PCR assay was developed for specific identification of *S.* Enteritidis. The HRM assay developed in combination with an *S.* Enteritidis-specific PCR can represent a useful protocol for accurate, cost-effective subtyping of 39 *Salmonella* serotypes.

DNA gyrase is encoded by *gyr*A and *gyr*B genes, while topoisomerase IV by *parC* and *par*E genes. Mutations in quinolone-resistant determining region (QRDR) in these genes are associated with quinolone-resistance (94), and sequencing represents the gold standard for detecting genetic changes. Ngoi and Thong (97) developed a HRM assay to screen for mutations in QRDR of gyrase and topoisomerase IV genes. Mutants were discriminated from the wild-type strains based on the transition of the HRM curves of all target regions, proving to be an efficient and cost-effective preliminary step for QRDR mutations identification in *Salmonella*.

MLVA and HRM were used to differentiate between the allelic variants in 5 tandem repeat loci in 117 *S.* Typhimurium isolates in Thailand (98). Both methods allowed the identification of 43 different genotypes, and slight differences in cluster analysis results were observed, showing that HRM based on allelic diversity at tandem repeat loci can represent a reliable and rapid method for differentiating *S.* Typhimurium isolates, and tracking sources of contamination.

Shigella sonnei

S. sonnei causes shigellosis, and consists of four specific lineages, and multidrug-resistant clones belonging to lineage III are an emerging global problem (99).

Sangal and co-workers (100) analyzed 68 *S. sonnei* isolates using HRM to test the phylogenetic robustness of 97 SNPs. HRM-based results of six chromosomal informative SNPs identified the major lineages/sublineages, demonstrating that *S. sonnei* phylogeny can be accurately defined with limited SNPs. More recently, Mazi and co-workers (101) proposed the same typing scheme, analyzing two *S. sonnei* strains for each lineage/sublineage,

and HRM typing separated lineages I, II, and III based on the analysis of fragments targeting SNPs within *kdu*D (2-deoxy-D-gluconate 3-dehydrogenase), *deo*A (thymidine phosphorylase), and *emr*A (multidrug resistance secretion protein), and enabled to distinguish sublineages IIIa, IIIb, and IIIc with the analysis of *fd*X (ferredoxin, electron carrer protein) and *men*F (menaquinone-specific isochorismate synthase) amplified products.

Vibrio cholerae

Cholera is a water-borne diarrheal disease caused by *V. cholerae* serogroup O1 and O139 (102). Recently, 108 *V. cholerae* strains were genotyped by melting curve-based multilocus melt typing based on seven housekeeping genes (103). Typing was also performed by MLST and PFGE. A total of 28 Tm values were grouped according to 7 housekeeping genes to obtain the code set of allelic gene, and classified into 18 types (Simpson's Index of Diversity=0.723). Sequences of genes polymorphism areas were clustered into the same 18 types with MLST reference method. With HRM, 46 strains, each representing a different PFGE type, were classified into 13 types (Simpson's Index of Diversity=0.614) and in A-K groups at 85% similarity (Simpson's Index of Diversity=0.858) with PFGE, indicating a comparable resolution with MLST and PFGE methods.

Yersinia enterocolitica, Y. pesudotuberculosis and Y. pestis

Y. enterocolitica, *Y. pseudotuberculosis* and *Y. pestis* are virulent to both humans and animals, and represent the causative agents of yersiniosis, a foodborne zoonosis, which is of substantial importance for public health (104, 105).

Souza and Falcão (106) proposed a method for *Y. enterocolitica* genotyping based on HRM to determine SNPs and establish the genetic diversity of 50 cultures. The isolates were clustered into three groups consistent with the pathogenic profile of each biotype.

Souza and Falcão (10) also developed HRM for Y. *pseudotuberculosis* genotyping based on SNPs differences. Ten SNPs (covering nine fragments) were screened from sequences within *16S rRNA*, *gln*A (glutamine synthetase), *gyr*B and *rec*A (recombinase) sequences of reference strains. A total of 40 clinical strains were analyzed, and MLST was used for comparison. Different melting profiles were found in five out of nine analyzed fragments. The phylogenetic tree demonstrated that *Y. pseudotuberculosis* strains were separated into two groups, with the first cluster including strains of 1/O:1a serogroup, and the second with 2/O:3 strains. The separation into two clusters based on bio-serogroups was consistent with the MLST database results.

Ciammaruconi and co-workers (107) described an allele variant discrimination method for *Y. pestis* based on HRM analysis. The isolates were genotyped analyzing the allele variants obtained for 25 VNTR loci. Fourteen loci were distinguishable and correctly assigned the alleles in a robust and reproducible way.

Discussion and Conclusions

Among nucleic acid-based methods, HRM has been widely used by several disciplines, providing simple and flexible solutions for characterization of variants, and addressing the needs of research laboratories of rapid turnaround times and minimal cost (108). HRM analysis involves amplification by real-time PCR of the target of interest (template) in the presence of a saturating dye, subsequent melting of the amplicons, and data analysis and interpretation (109). As a relatively new technique, HRM represents a versatile genetic tool for general use, with the main application of gene scanning (110),

and an alternative to approaches requiring separations or labeled probes. Since the melting profile depends on the GC content, length, sequence and strand complementarity of the product, HRM analysis is highly suitable for the detection of single-base variants and small insertions or deletions (109). In processing HRM data, both melting temperature shifts and curve shape are used to identify sequence differences; homozygous allelic/sequence variants are typically characterized by the temperature shift observed in an HRM melt curve, whereas heterozygotes are commonly characterized by a change in melt curve shape generated from base-pairing mismatches. HRM data are often plotted using a difference curve to magnify the melt profile differences between diverse clusters of the same genotype.

The aims of this review were to summarize and analyze the applications of HRM for genotyping of bacterial pathogens, to highlight the potential advantages, and to address future epidemiological research studies. The literature search allowed the conclusion that HRM has been successfully applied to both Gram-positive and Gram-negative pathogens and used for different approaches. In summary, the method was used: a) to evaluate genetic diversity of bacterial isolates and to subtype them at species/subspecies level, based also on allele discrimination/identification and mutations screening; b) to recognize phylogenetic groupings (lineage, sublineage, subgroups); c) to identify antibiotic resistant and susceptible isolates; d) to detect and screen for mutations related to drug-resistance; e) to discriminate gene isoforms.

Although at the state of art MLST and PFGE often represent the standard methods for resolving the bacterial population genetics (111, 112), these techniques are expensive and time consuming. In details, the estimated reagent cost per sample for PFGE is € 22–27.00, with an approximated time to obtain results of 4 days (113), while the cost of the MLST-based scheme for SNPs analysis with HRM (Minim typing) is 10–20% of MLST (approximately € 70.00 per sample by testing 7 loci), and is performed in a single-step, enabling low cost surveillance and rapid response in case of outbreaks (84). Furthermore, although WGS is becoming increasingly applied, the cost is approximately € 1,000 per genome, or more.

In this review, HRM was shown to be applicable to characterize genetic elements in a wide range of bacterial species, and, in most of instances, it was possible to achieve excellent typeability and discriminatory power, with high concordance of typing results obtained with gold standard or alternative methods. Moreover, HRM assays were used to evaluate genetic determinants associated to antibiotic-resistance or to screen for associated mutations in key gene fragments, but did not always yield the same accuracy and results of standard phenotypic procedures; sensitivity and specificity were not optimal because in some instances targeted amplicons did not encompass all the crucial mutations. As underlined in the reviewed studies, the HRM approach is relatively new, and has been applied to diverse research fields of infectious diseases. Despite the introduction of sequencing-based methods, HRM is characterized by major advantages, including the low cost, utilization of existing equipment, rapidity, flexibility, and versatility (1, 109).

However, some limitations affect this technology, and key aspects in the development of an HRM assay should be carefully considered. First, a specific and efficient amplification of the template is a prerequisite for reliable and reproducible melting profiles. Second, important concerns are related to the targeted fragments (type and size), primer design and selection, as well as the optimization of PCR conditions, which all represent crucial steps in HRM-based assays development (109). General guidelines must be followed in designing primers, with specific focus on avoiding the formation of

non-specific products or dimers. Moreover, the length of the targeted amplicons can significantly affect the sensitivity of HRM analysis, which should not exceed 300 base pairs: if the amplicon is longer, any difference in the melting curves caused by small sequence variations will be difficult to be distinguished and evaluated. In addition, long amplicons may contain several melting domains, resulting in rather complex melting profiles (109). The melting temperature is also typically higher for DNA fragments longer and/or with a high GC content that generally should be 30–80%, with maximum of 2 G or C nucleotides in the last 5 at 3' end.

Despite the high efficiency in mutation screening, HRM assay can detect only the presence of base substitutions, and the melting profiles should not be used directly for variant classification without validation by DNA sequencing. False positive and negative may occur due to synonymous mutations and, for example, mutations targeting other regions or resistance caused by uncharacterized mechanisms will not be identified. Hence, despite the usefulness of the method, sequencing and phenotypic analysis represent the ultimate proof for mutations leading to amino acid changes and alteration of protein structure (114).

PCR conditions (i.e., temperature gradient selection of the annealing temperature, $MgCl_2$ concentration, etc.) should be carefully optimized, influencing the melting behaviour of dsDNA. An efficient amplification is indicated by low threshold cycles and amplification curves completely reaching the plateau (109). Other aspects affecting the performance of HRM assays include DNA isolation and preparation, the quality and quantity of DNA template (high integrity and purity) (109), and the samples to test should not differ in target DNA concentrations to achieve similar threshold cycles values (13, 110).

In conclusion, the most common applications of HRM method for bacterial typing identified from this literature review are related to SNP-based genotyping with the analysis of gene fragments within the MLST loci, following an approach termed mini-MLST or Minim typing (84). HRM is particularly suitable for generation of data, which are interpretable in terms of the MLST database of a relevant species, based on specific steps as follows: SNPs derived by MLST data based on the maximal Simpson's Index of Diversity; amplification of fragments between 50 bp and 150 bp incorporating these SNPs and nearby SNPs; use of computational approaches to interpret HRM data in terms of the MLST database (84). Although the resolving power is not totally correspondent to MLST, the Simpson's Index of Diversity provided by HRM method, as underlined by many reviewed studies, is typically >0.95.

Hence, HRM technology has a great potential for the genetic characterization of a wide range of pathogenic microbes, strongly facilitates research studies, and provides rapid screening for epidemiological surveillance.

Acknowledgements
We acknowledge the helpful comments from Dr. Jim McLauchlin, Public Health England, London, UK.
Competing interests
Authors do not have any conflict of interests.

Riassunto

High Resolution Melting come metodica emergente rapida, affidabile, accurata ed economica per la genotipizzazione di batteri patogeni e per l'implementazione della sorveglianza epidemiologica-molecolare: una revisione della letteratura

Introduzione. I metodi di tipizzazione molecolare sono essenziali per il rilevamento di focolai epidemici, per monitorare l'evoluzione e le dinamiche delle popolazioni microbiche, quindi, per una efficace sorveglianza epidemiologica delle malattie infettive. A partire dal 2002, l'introduzione di un metodo basato sull'analisi della temperatura di melting dei prodotti amplificati, noto come High Resolution Melting (HRM), ha trovato

numerose applicazioni negli studi epidemiologici, sia per l'identificazione delle specie batteriche, sia per la tipizzazione molecolare, così come un ampio utilizzo in molti ambiti di ricerca. Il metodo HRM si basa sull'uso di molecole intercalanti di terza generazione, di piattaforme avanzate di real-time PCR e strumenti bioinformatici per l'analisi dei dati.

Obiettivo. In questo studio, attraverso una revisione sistematica della letteratura, sono descritti l'uso, l'applicazione e l'utilità dell'HRM per la genotipizzazione dei batteri patogeni nel contesto della sorveglianza epidemiologica in Sanità Pubblica.

Materiali e metodi. La revisione della letteratura è stata condotta nei mesi di luglio e agosto 2016, consultando le principali banche dati: PubMed/Medline, Scopus, EMBASE e ISI Web of Science. La strategia di ricerca è stata eseguita secondo le seguenti parole chiave: high resolution melting e batteri e genotipizzazione o tipizzazione molecolare. Sono stati, quindi, selezionati ed esaminati tutti gli articoli che avevano valutato l'uso dell'HRM per la genotipizzazione di batteri patogeni, tenendo conto dell'obiettivo di ogni studio analizzato, il razionale per l'utilizzo di tale tecnica ed i principali risultati ottenuti rispetto ai metodi considerati "gold standard" e/o altri metodi alternativi, quando disponibili.

Risultati. Il metodo HRM è stato ampiamente utilizzato per la tipizzazione molecolare sia di batteri patogeni Gram-positivi, sia Gram-negativi, rappresentando una metodologia molecolare versatile ed utile: a) per valutare la diversità genetica degli isolati e per tipizzare a livello di specie e sottospecie, anche sulla base della discriminazione allelica e/o dell'identificazione di mutazioni puntiformi; b) per riconoscere gruppi filogenetici (lineage, sublineage, sottogruppi); c) per determinare la resistenza agli antimicrobici; d) per identificare mutazioni legate alla resistenza ai farmaci; e) per discriminare isoforme geniche. L'HRM ha mostrato, in quasi tutti i casi, un'eccellente tipizzabilità e un ottimo potere discriminante, con un'elevata concordanza dei risultati di tipizzazione ottenuti con metodi "gold standard" o altri approcci. Tuttavia, nella valutazione dei determinanti genetici associati alla resistenza agli antibiotici o per lo screening di mutazioni ad essa associate, la sensibilità e la specificità non sono sempre state ottimali, poichè gli ampliconi non sempre comprendevano le mutazioni cruciali.

Conclusioni. Nonostante la recente introduzione di metodi basati sul sequenziamento, l'HRM merita una notevole considerazione nello studio dell'epidemiologia molecolare delle malattie infettive, essendo caratterizzato da basso costo, rapidità, flessibilità e versatilità. Tuttavia, ci sono alcune limitazioni nello sviluppo dei saggi che, comunque, devono essere considerate con attenzione. L'applicazione più comune dell'HRM per tipizzare i batteri si riferisce alla genotipizzazione basata su mutazioni puntiformi (Single Nucleotide Polymorphisms, SNPs) attraverso l'analisi di frammenti genici di loci utilizzati nella tecnica multilocus sequence typing (MLST), seguendo un approccio chiamato mini-MLST o Minim-typing. Anche se il potere di risoluzione non è del tutto corrispondente a quello ottenuto con l'MLST, l'indice del potere discriminante fornito dall'HRM in genere è stato > 0,95. Inoltre, il costo di tale approccio è inferiore all'MLST, consentendo di effettuare una sorveglianza a costi ridotti e allo stesso tempo fornendo risposte rapide in caso di focolai epidemici. Pertanto, il potenziale della tecnologia HRM può fortemente facilitare lo studio dell'epidemiologia molecolare delle malattie infettive e semplificare la caratterizzazione dei batteri patogeni.

References

1. Ruskova L, Raclavsky V. The potential of high resolution melting analysis (hrma) to streamline, facilitate and enrich routine diagnostics in medical microbiology. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2011; **155**(3): 239-52.
2. Amar CFL, Tamburro M, Dear P, Martin K, Grant K. Use of High Resolution Melting technique for fingerprint Salmonella Genomic Island-1 (SGI-1) and *Listeria monocytogenes* virulence genes. In: Proceedings of the Annual Health Protection Agency Meeting Conference, Warwick University. Coventry, UK, September 14-16, 2009.
3. Amar CFL, Tamburro M, Dear P, Grant K. Virulotyping of L*isteria monocytogenes* by high resolution melt analysis. In: Proceedings of the ISOPOL XVII, International Symposium on Problems of Listeriosis. Porto, Portugal, May 5-8, 2010.
4. Tamburro M, Grant K, Amar C, Pontello M, Sammarco ML, Ripabelli G. High Resolution Melting Analysis (HRMA) per la caratterizzazione molecolare di LIPI-1 e inlAB in *Listeria monocytogenes*. Proceedings of the VII Workshop Nazionale Sistema di sorveglianza delle infezioni enteriche ENTER-NET Italia "Infezioni trasmesse da alimenti e acqua: diagnostica ed epidemiologia". Roma, 4-5 novembre 2009.
5. Derzelle S. Single-nucleotide polymorphism discrimination using high-resolution melting analysis for the genotyping of *Bacillus anthracis*. Methods Mol Biol 2015; **1247**: 361-71.
6. Martino A, Mancuso T, Rossi AM. Application of high-resolution melting to large-scale, high-

throughput SNP genotyping: a comparison with the TaqMan method. J Biomol Screen 2010; **15**(6): 623-9.
7. Choi GE, Lee SM, Yi J, et al. High-resolution melting curve analysis for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates. J Clin Microbiol 2010; **48**(11): 3893-8.
8. Lilliebridge RA, Tong SY, Giffard PM, Holt DC. The utility of high-resolution melting analysis of SNP nucleated PCR amplicons--an MLST based *Staphylococcus aureus* typing scheme. PLoS One 2011; **6**(6): e19749.
9. Tong SY, Giffard PM. Microbiological applications of high-resolution melting analysis. J Clin Microbiol 2012; **50**(11): 3418-21.
10. Souza RA, Falcão JP. A novel high-resolution melting analysis-based method for *Yersinia pseudotuberculosis* genotyping. J Microbiol Methods 2012; **91**(3): 329-35.
11. Derzelle S, Mendy C, Laroche S, Madani N. Use of high-resolution melting and melting temperature-shift assays for specific detection and identification of *Bacillus anthracis* based on single nucleotide discrimination. J Microbiol Methods 2011; **87**(2): 195-201.
12. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003; **49**(6 Pt 1): 853-60.
13. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics 2007; **8**(6): 597-608.
14. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 2003; **49**(3): 396-406.
15. Sammarco ML, Ripabelli G, Tamburro M. Epidemiologia molecolare delle malattie infettive: metodi di analisi ed interpretazione dei risultati. Ann Ig 2014; **26**(1): 10-45.
16. Merchant-Patel S, Blackall PJ, Templeton J, et al. *Campylobacter jejuni* and *Campylobacter coli* genotyping by high-resolution melting analysis of a flaA fragment. Appl Environ Microbiol 2010; **76**(2): 493-99.
17. Tong SY, Lilliebridge RA, Holt DC, McDonald MI, Currie BJ, Giffard PM. High-resolution melting analysis of the spa locus reveals significant diversity within sequence type 93 methicillin-resistant *Staphylococcus aureus* from northern Australia. Clin Microbiol Infect 2009; **15**(12): 1126-31.
18. Goel AK. Anthrax: A disease of biowarfare and public health importance. World J Clin Cases 2015; **3**(1): 20-33.
19. Keim P, Van Ert MN, Pearson T, Vogler AJ, Huynh LY, Wagner DM. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect Genet Evol 2004; **4**(3): 205-13.
20. Fortini D, Ciammaruconi A, De Santis R, et al. Optimization of high-resolution melting analysis for low-cost and rapid screening of allelic variants of *Bacillus anthracis* by multiple-locus variable-number tandem repeat analysis. Clin Chem 2007; **53**(7): 1377-80.
21. Derzelle S, Laroche S, Le Flèche P, et al. Characterization of genetic diversity of *Bacillus anthracis* in France by using high-resolution melting assays and multilocus variable-number tandem-repeat analysis. J Clin Microbiol. 2011; **49**(12): 4286-92.
22. Fehér C, Mensa J. A Comparison of Current Guidelines of Five International Societies on *Clostridium difficile* Infection Management. Infect Dis Ther 2016; **5**(3): 207-30.
23. Cózar-Llistó A, Ramos-Martinez A, Cobo J. *Clostridium difficile* Infection in Special High-Risk Populations. Infect Dis Ther 2016; **5**(3): 253-69.
24. Zacharioudakis IM, Zervou FN, Pliakos EE, Ziakas PD, Mylonakis E. Colonization with toxinogenic *C. difficile* upon hospital admission, and risk of infection: a systematic review and meta-analysis. Am J Gastroenterol 2015; **110**(3): 381-90; quiz 391.
25. Martin JS, Monaghan TM, Wilcox MH. *Clostridium difficile* infection: epidemiology, diagnosis and understanding transmission. Nat Rev Gastroenterol Hepatol 2016; **13**(4): 206-16.
26. Pecavar V, Blaschitz M, Hufnagl P, et al. High-resolution melting analysis of the single nucleotide polymorphism hot-spot region in the rpoB gene as an indicator of reduced susceptibility to rifaximin in *Clostridium difficile*. J Med Microbiol 2012; **61**(Pt 6): 780-5.
27. de Almeida Monteiro A, Pires RN, Persson S, Rodrigues Filho EM, Pasqualotto AC. A search for *Clostridium difficile* ribotypes 027 and 078

in Brazil. Braz J Infect Dis 2014; **18**(6): 672-4.
28. Guzman Prieto AM, van Schaik W, Rogers MR, et al. Global Emergence and Dissemination of Enterococci as Nosocomial Pathogens: Attack of the Clones? Front Microbiol 2016; **7**: 788.
29. Tong SY, Xie S, Richardson LJ, et al. High-resolution melting genotyping of *Enterococcus faecium* based on multilocus sequence typing derived single nucleotide polymorphisms. PLoS One 2011; **6**(12): e29189.
30. Alonso M, Marín M, Iglesias C, Cercenado E, Bouza E, García de Viedma D. Rapid identification of linezolid resistance in *Enterococcus* spp. based on high-resolution melting analysis. J Microbiol Methods 2014; **98**: 41-3.
31. Lomonaco S, Nucera D, Filipello V. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infect Genet Evol 2015; **35**: 172-83.
32. Tamburro M, Sammarco ML, Ammendolia MG, Fanelli I, Minelli F, Ripabelli G. Evaluation of transcription levels of inlA, inlB, hly, bsh and prfA genes in *Listeria monocytogenes* strains using quantitative reverse-transcription PCR and ability of invasion into human CaCo-2 cells. FEMS Microbiol Lett 2015; **362**(6): pii: fnv018.
33. Tamburro M, Ripabelli G, Vitullo M, et al. Gene expression in *Listeria monocytogenes* exposed to sublethal concentration of benzalkonium chloride. Comp Immunol Microbiol Infect Dis 2015; **40**: 31-9.
34. Tamburro M, Ripabelli G, Fanelli I, Grasso GM, Sammarco ML. Typing of *Listeria monocytogenes* strains isolated in Italy by inlA gene characterisation and evaluation of a new cost-effective approach to antisera selection for serotyping. J Appl Microbiol 2010; **108**(5): 1602-11.
35. Vitullo M, Grant KA, Sammarco ML, Tamburro M, Ripabelli G, Amar CF. Real-time PCRs assay for serogrouping *Listeria monocytogenes* and differentiation from other *Listeria* spp. Mol Cell Probes 2013; **27**(1): 68-70.
36. Pietzka AT, Stöger A, Huhulescu S, Allerberger F, Ruppitsch W. Gene Scanning of an Internalin B Gene Fragment Using High-Resolution Melting Curve Analysis as a Tool for Rapid Typing of *Listeria monocytogenes*. J Mol Diagn 2011; **13**(1): 57-63.
37. Sakaridis I, Ganopoulos I, Madesis P, Tsaftaris A, Argiriou A. Genotyping of *Listeria monocytogenes* isolates from poultry carcasses using high resolution melting (HRM) analysis. Biotechnol Biotechnol Equip 2014 2; **28**(1): 107-11.
38. Stefani S, Chung DR, Lindsay JA, et al. Meticillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. Int J Antimicrob Agents 2012; **39**(4); 273-82.
39. Carmona-Torre F, Rua M, Del Pozo JL. Directed therapeutic approach to *Staphylococcus aureus* infections. Clinical aspects of prescription. Rev Esp Quimioter 2016; **29**(Suppl 1): 15-20.
40. Foster TJ. Immune evasion by staphylococci. Nat Rev Microbiol 2005; **3**(12): 948-58.
41. Votintseva AA, Fung R, Miller RR, et al. Prevalence of *Staphylococcus aureus* protein A (spa) mutants in the community and hospitals in Oxfordshire. BMC Microbiol 2014; **14**: 63.
42. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. Antimicrob Agents Chemother 2009; **53**(12): 4961-7.
43. Stephens AJ, Inman-Bamber J, Giffard PM, Huygens F. High-resolution melting analysis of the spa repeat region of *Staphylococcus aureus*. Clin Chem 2008; **54**(2): 432-6.
44. Chen JH, Cheng VC, Chan JF, et al. The use of high-resolution melting analysis for rapid spa typing on methicillin-resistant *Staphylococcus aureus* clinical isolates. J Microbiol Methods 2013; **92**(2): 99-102.
45. Mazi W, Sangal V, Sandstrom G, Saeed A, Yu J. Evaluation of spa-typing of methicillin-resistant *Staphylococcus aureus* using high-resolution melting analysis. Int J Infect Dis 2015; **38**: 125-8.
46. Mayerhofer B, Stöger A, Pietzka AT, et al. Improved protocol for rapid identification of certain spa types using high resolution melting curve analysis. PLoS One 2015; **10**(3): e0116713.
47. Mongelli G, Bongiorno D, Agosta M, Benvenuto S, Stefani S, Campanile F. High Resolution Melting-Typing (HRMT) of methicillin-resistant *Staphylococcus aureus* (MRSA): The new frontier to replace multi-locus sequence typing (MLST) for epidemiological surveillance studies. J Microbiol Methods 2015; **117**: 136-8.
48. Tong SY, Lilliebridge RA, Holt DC, Coombs GW, Currie BJ, Giffard PM. Rapid detection of

H and R Panton-Valentine leukocidin isoforms in *Staphylococcus aureus* by high-resolution melting analysis. Diagn Microbiol Infect Dis 2010; **67**(4): 399-401.
49. Jeremiah CJ, Kandiah JP, Spelman DW, et al. Differing epidemiology of two major healthcare-associated methicillin-resistant *Staphylococcus aureus clones*. J Hosp Infect 2016; **92**(2): 183-90.
50 Zumla A, Raviglione M, Hafner R, von Reyn CF. Tuberculosis. N Eng J Med 2013; **368**; 745-55.
51. Unissa AN, Subbian S, Hanna LE, Selvakumar N. Overview on mechanisms of isoniazid action and resistance in *Mycobacterium tuberculosis*. Infect Genet Evol 2016. pii: S1567-1348-(16)30387-2.
52. Pietzka AT, Indra A, Stöger A, et al. Rapid identification of multidrug-resistant *Mycobacterium tuberculosis* isolates by rpoB gene scanning using high-resolution melting curve PCR analysis. J Antimicrob Chemother 2009; **63**(6): 1121-7.
53. Ramirez MV, Cowart KC, Campbell PJ, et al. Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* by use of real-time PCR and high-resolution melt analysis. J Clin Microbiol 2010; **48**(11): 4003-9.
54. Ong DC, Yam WC, Siu GK, Lee AS. Rapid detection of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* by high-resolution melting analysis. J Clin Microbiol 2010; **48**(4): 1047-54.
55. Chen X, Kong F, Wang Q, Li C, Zhang J, Gilbert GL. Rapid detection of isoniazid, rifampin, and ofloxacin resistance in *Mycobacterium tuberculosis* clinical isolates using high-resolution melting analysis. J Clin Microbiol 2011; **49**(10): 3450-7.
56. Yadav R, Sethi S, Mewara A, Dhatwalia SK, Gupta D, Sharma M. Rapid detection of rifampicin, isoniazid and streptomycin resistance in *Mycobacterium tuberculosis* clinical isolates by high-resolution melting curve analysis. J Appl Microbiol 2012; **113**(4): 856-62.
57. Lee AS, Ong DC, Wong JC, Siu GK, Yam WC. High-resolution melting analysis for the rapid detection of fluoroquinolone and streptomycin resistance in *Mycobacterium tuberculosis*. PLoS One 2012; **7**(2): e31934.
58. Nour MS, El-Shokry MH, Shehata IH, Abd-El Aziz AM. Evaluation of rezasurin microtiter assay and high resolution melting curve analysis for detection of rifampicin and isoniazid resistance of *Mycobacterium tuberculosis* clinical isolates. Clin Lab 2013; **59**(7-8): 763-71.
59. Hong CY, Wang F, Liu XL. Detection of pncA mutation associated with pyrazinamide resistance in *Mycobacterium tuberculosis* by high-resolution melting cure analysis. Zhonghua Jie He He Hu Xi Za Zhi 2013; **36**(3): 198-201.
60. Nagai Y, Iwade Y, Hayakawa E, et al. High resolution melting curve assay for rapid detection of drug-resistant *Mycobacterium tuberculosis*. J Infect Chemother 2013; **19**(6): 1116-25.
61. Haeili M, Fooladi AI, Bostanabad SZ, Sarokhalil DD, Siavoshi F, Feizabadi MM. Rapid screening of rpoB and katG mutations in *Mycobacterium tuberculosis* isolates by high-resolution melting curve analysis. Indian J Med Microbiol 2014; **32**(4): 398-403.
62. Watcharasamphankul W, Houpt ER, Foongladda S. Rapid detection of pyrazinamide resistant *Mycobacterium tuberculosis* by high resolution melting curve analysis. J Med Assoc Thai 2013; **96**(9): 1218-23.
63. Malhotra B, Goyal S, Bhargava S, Reddy PV, Chauhan A, Tiwari J. Rapid detection of rifampicin resistance in *Mycobacterium tuberculosis* by high-resolution melting curve analysis. Int J Tuberc Lung Dis 2015; **19**(12): 1536-41.
64. Otto M. *Staphylococcus epidermidis*--the 'accidental' pathogen. Nat Rev Microbiol 2009; **7**(8): 555-67.
65. Rogers KL, Fey PD, Rupp ME. Coagulase-negative staphylococcal infections. Infect Dis Clin North Am 2009; **23**(1): 73-98.
66. Gabriel EM, Douarre PE, Fitzgibbon S, et al. High-resolution melting analysis for rapid detection of linezolid resistance (mediated by G2576T mutation) in *Staphylococcus epidermidis*. J Microbiol Methods 2012; **90**(2): 134-6.
67. Bessen DE, McShan WM, Nguyen SV, Shetty A, Agrawal S, Tettelin H. Molecular epidemiology and genomics of group A Streptococcus. Infect Genet Evol 2015; **33**: 393-418.
68. Excler JL, Kim JH. Accelerating the development of a group A Streptococcus vaccine: an urgent public health need. Clin Exp Vaccine Res 2016; **5**(2): 101-7.
69. Slinger R, Desjardins M, Moldovan I, Harvey SB, Chan F. A rapid, high-resolution melting (HRM) multiplex PCR assay to detect macrolide resistance determinants in group A streptococcus. Int J Antimicrob Agents 2011; **38**(2): 183-5.

70. Richardson LJ, Tong SY, Towers RJ, et al. Preliminary validation of a novel high-resolution melt-based typing method based on the multilocus sequence typing scheme of *Streptococcus pyogenes*. Clin Microbiol Infect 2011; **17**(9): 1426-34.
71. Bidet P, Liguori S, Plainvert C, et al. Identification of group A streptococcal emm types commonly associated with invasive infections and antimicrobial resistance by the use of multiplex PCR and high-resolution melting analysis. Eur J Clin Microbiol Infect Dis 2012; **31**(10): 2817-26.
72. Kilgore PE, Salim AM, Zervos MJ, Schmitt HJ. Pertussis: Microbiology, Disease, Treatment, and Prevention. Clin Microbiol Rev 2016; **29**(3): 449-86.
73. Zhang Q, Li M, Wang L, Xin T, He Q. High-resolution melting analysis for the detection of two erythromycin-resistant *Bordetella pertussis* strains carried by healthy schoolchildren in China. Clin Microbiol Infect 2013; **19**(6): E260-2.
74. Ripabelli G, Tamburro M, Minelli F, Leone A, Sammarco ML. Prevalence of virulence-associated genes and cytolethal distending toxin production in *Campylobacter* spp. isolated in Italy. Comp Immunol Microbiol Infect Dis 2010; **33**(4): 355-64.
75. Fitzgerald C. *Campylobacter*. Clin Lab Med 2015; **35**(2): 289-98.
76. Sammarco ML, Ripabelli G, Fanelli I, Grasso GM, Tamburro M. Prevalence and biomolecular characterization of *Campylobacter* spp. isolated from retail meat. J Food Prot 2010; **73**(4): 720-8.
77. Lévesque S, Michaud S, Arbeit RD, Frost EH. High-resolution melting system to perform multilocus sequence typing of *Campylobacter jejuni*. PLoS One 2011; **6**(1): e16167.
78. Gomes CN, Souza RA, Passaglia J, Duque SS, Medeiros MI, Falcão JP. Genotyping of *Campylobacter coli* strains isolated in Brazil suggests possible contamination amongst environmental, human, animal and food sources. J Med Microbiol 2016; **65**(1): 80-90.
79. Ziklo N, Huston WM, Hocking JS, Timms P. *Chlamydia trachomatis* Genital Tract Infections: When Host Immune Response and the Microbiome Collide. Trends Microbiol 2016; **24**(9): 750-65.
80. Li JH, Yin YP, Zheng HP, et al. A high-resolution melting analysis for genotyping urogenital *Chlamydia trachomatis*. Diagn Microbiol Infect Dis 2010; **68**(4): 366-74.
81. Parsons BD, Zelyas N, Berenger BM, Chui L. Detection, Characterization, and Typing of Shiga Toxin-Producing *Escherichia coli*. Front Microbiol 2016; **7**: 478.
82. Etoh Y, Hirai S, Ichihara S, et al. Evolutionary model of the divergence of enterohemorrhagic *Escherichia coli* O157 lineage I/II clades reconstructed from high resolution melting and Shiga-like toxin 2 analyses. Infect Genet Evol 2014; **24**: 140-5.
83. Girmenia C, Serrao A, Canichella M. Epidemiology of Carbapenem Resistant *Klebsiella pneumoniae* Infections in Mediterranean Countries. Mediterr J Hematol Infect Dis 2016; **8**(1): e2016032.
84. Andersson P, Tong SY, Bell JM, Turnidge JD, Giffard PM. Minim typing--a rapid and low cost MLST based typing tool for *Klebsiella pneumoniae*. PLoS One 2012; **7**(3): e33530.
85. Roth AL, Hanson ND. Rapid detection and statistical differentiation of KPC gene variants in Gram-negative pathogens by use of high-resolution melting and ScreenClust analyses. J Clin Microbiol 2013; **51**(1): 61-5.
86. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev 2009; **22**(1): 161-82.
87. Geyer CN, Hanson ND. Multiplex high-resolution melting analysis as a diagnostic tool for detection of plasmid-mediated AmpC β-lactamase genes. J Clin Microbiol 2014; **52**(4): 1262-5.
88. Adler B, de la Peña Moctezuma A. Leptospira and leptospirosis. Vet Microbiol 2010; **140**(3-4): 287-96.
89. Naze F, Desvars A, Picardeau M, Bourhy P, Michault A. Use of a New High Resolution Melting Method for Genotyping Pathogenic *Leptospira* spp. PLoS One 2015; **10**(7): e0127430.
90. Fujitani S, Sun HY, Yu VL, Weingarten JA. Pneumonia due to *Pseudomonas aeruginosa*: part I: epidemiology, clinical diagnosis, and source. Chest 2011; **139**(4): 909-19.
91. Naze F, Jouen E, Randriamahazo RT, et al. *Pseudomonas aeruginosa* outbreak linked to mineral water bottles in a neonatal intensive care unit: fast typing by use of high-resolution melting analysis of a variable-number tandem-repeat locus. J Clin Microbiol 2010; **48**(9): 3146-52.
92. Anuj SN, Whiley DM, Kidd TJ, et al. Rapid single-nucleotide polymorphism-based iden-

tification of clonal *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis by the use of real-time PCR and high-resolution melting curve analysis. Clin Microbiol Infect 2011; **17**(9): 1403-8.

93. Bell RL, Jarvis KG, Ottesen AR, McFarland MA, Brown EW. Recent and emerging innovations in Salmonella detection: a food and environmental perspective. Microb Biotechnol 2016; **9**(3): 279-92.
94. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003; **51**(5): 1109-17.
95. Slinger R, Bellfoy D, Desjardins M, Chan F. High-resolution melting assay for the detection of gyrA mutations causing quinolone resistance in *Salmonella enterica* serovars Typhi and Paratyphi. Diagn Microbiol Infect Dis 2007; **57**(4): 455-8.
96. Zeinzinger J, Pietzka AT, Stöger A, et al. One-step triplex high-resolution melting analysis for rapid identification and simultaneous subtyping of frequently isolated Salmonella serovars. Appl Environ Microbiol 2012; **78**(9): 3352-60.
97. Ngoi ST, Thong KL. High resolution melting analysis for rapid mutation screening in gyrase and Topoisomerase IV genes in quinolone-resistant *Salmonella enterica*. Biomed Res Int 2014; 2014: 718084.
98. Keeratipibul S, Silamat P, Phraephaisarn C, et al. Genotyping of *Salmonella enterica* serovar Typhimurium isolates by multilocus variable number of tandem repeat high-resolution melting analysis (MLV-HRMA). Foodborne Pathog Dis 2015; **12**(1): 8-20.
99. Holt KE, Baker S, Weill FX, et al. *Shigella sonnei* genome sequencing and phylogenetic analysis indicate recent global dissemination from Europe. Nat Genet 2012; **44**(9): 1056-9.
100. Sangal V, Holt KE, Yuan J, et al. Global phylogeny of *Shigella sonnei* strains from limited single nucleotide polymorphisms (SNPs) and development of a rapid and cost-effective SNP-typing scheme for strain identification by high-resolution melting analysis. J Clin Microbiol 2013; **51**(1): 303-5.
101. Mazi W, Sangal V, Saeed A, Sandstrom G, Weill FX, Yu J. Rapid Genotyping of *Shigella sonnei* by Use of Multiplex High-Resolution Melting. J Clin Microbiol 2015; **53**(7): 2389-91.
102. Mercogliano F, Vitullo M, Tamburro M, et al. *Vibrio* spp. infections of clinical significance and implication for public health. Ann Ig 2012; **24**(1): 85-102.
103. Huang S, Liu Z, Wen H, Li L, Li Q, Huang J. Development of a rapid molecular typing method for *Vibrio cholerae* using melting curve-based multilocus melt typing. Zhonghua Yu Fang Yi Xue Za Zhi 2015; **49**(2): 122-7.
104. Ripabelli G, Sammarco ML, Fanelli I, Grasso GM. Detection of *Salmonella*, *Listeria* spp., *Vibrio* spp., and *Yersinia enterocolitica* in frozen seafood and comparison with enumeration for faecal indicators: implication for public health. Ann Ig 2004; **16**(4): 531-9.
105. Bancerz-Kisiel A, Szweda W. Yersiniosis - a zoonotic foodborne disease of relevance to public health. Ann Agric Environ Med 2015; **22**(3): 397-402.
106. Souza RA, Falcão JP. A novel high-resolution melting analysis-based method for *Yersinia enterocolitica* genotyping. J Microbiol Methods 2014; **106**: 129-34.
107. Ciammaruconi A, Grassi S, Faggioni G, et al. A rapid allele variant discrimination method for *Yersinia pestis* strains based on high-resolution melting curve analysis. Diagn Microbiol Infect Dis 2009; **65**(1): 7-13.
108. Montgomery JL, Sanford LN, Wittwer CT. High-resolution DNA melting analysis in clinical research and diagnostics. Expert Rev Mol Diagn 2010; **10**(2): 219-40.
109. Druml B, Cichna-Markl M. High resolution melting (HRM) analysis of DNA--its role and potential in food analysis. Food Chem 2014; **158**: 245-54.
110. Vossen RH, Aten E, Roos A, den Dunnen JT. High-resolution melting analysis (HRMA): more than just sequence variant screening. Hum Mutat 2009; **30**(6): 860-6.
111. Pérez-Losada M, Cabezas P, Castro-Nallar E, Crandall KA. Pathogen typing in the genomics era: MLST and the future of molecular epidemiology. Infect Genet Evol 2013; **16**:38-53.
112. Parizad EG, Parizad EG, Valizadeh A. The Application of Pulsed Field Gel Electrophoresis in Clinical Studies. J Clin Diagn Res 2016; **10**(1): DE01-4.
113. Dominguez SR, Anderson LI, Kotter CV et al. Comparison of Whole-Genome Sequencing and Molecular-Epidemiological Techniques for *Clostridium difficile* Strain Typing. J Pediatric Infect Dis Soc 2016; 5(3): 329-32.

114. Tindall EA, Petersen DC, Woodbridge P, Schipany K, Hayes VM. Assessing high-resolution melt curve analysis for accurate detection of gene variants in complex DNA fragments. Hum Mutat 2009; **30**(6): 876-83.

Corresponding Author: Prof. Giancarlo Ripabelli, Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, Via De Sanctis, 86100 Campobasso, Italy
e-mail: ripab@unimol.it