Reports

BioTechniques

Accurate, fast and cost-effective simultaneous detection of bacterial meningitis by qualitative PCR with high-resolution melting

Ivano de Filippis*,[1], Aline Carvalho de Azevedo[1], Irene de Oliveira Lima[1], Nicolle F Lima Ramos[1], Claudia Ferreira de Andrade[1] & Antonio ECC de Almeida[1]

*[1]Laboratório de Microrganismos de Referência, Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, 21040-900, Brazil; *Author for correspondence: ivano.defilippis@fiocruz.br*

BioTechniques 74: 99–106 (February 2023) 10.2144/btn-2022-0089

First draft submitted: 18 August 2022; Accepted for publication: 9 January 2023; Published online: 27 February 2023

ABSTRACT

Neisseria meningitidis, *Haemophilus influenzae* and *Streptococcus pneumoniae* are leading causes of meningitis and acute invasive infections. PCR-based methods are widely used for the diagnosis and surveillance of bacterial pathogens because of their high sensitivity, specificity and high-throughput capabilities compared with conventional laboratory methods. This study evaluated a high-resolution melting qualitative PCR analysis method for the simultaneous detection of these three pathogens. The assay has been optimized to detect three species-specific genes of each organism isolated from clinical samples, enabling accurate identification of the etiological agent. The method proved to be highly sensitive and cheaper than the real-time PCR TaqMan® system because it is probe-free; it could be used for the diagnosis of invasive diseases in public health laboratories of developing countries.

METHOD SUMMARY

A real-time qualitative PCR with high-resolution melting (qPCR-HRM) for simultaneous detection of the three most frequent bacteria causing meningitis was evaluated. The proposed method was shown to be as sensitive and specific as the gold-standard TaqMan qPCR, but overall the reagent costs of qPCR-HRM are about 20% of those needed for the TaqMan system because it does not require the use of fluorescent probes. This is the first report of the use of qPCR-HRM for rapid and cost-effective diagnostic of bacterial meningitis.

KEYWORDS:

bacterial meningitis ● invasive disease ● multiplex qPCR-HRM ● rapid diagnosis

Invasive bacterial disease is a severe illness and a serious public health burden, with high rates of morbidity and mortality worldwide. The most common causes of invasive bacterial disease associated with septicemia and meningitis are infection with *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* [1,2]. The WHO estimates that about 170,000 deaths occur annually from the disease worldwide; the case fatality rate can be as high as 50% if not treated [3]. In middle- and low-income countries, acute bacterial meningitis remains the fourth leading cause of disability [3,4].

The identification of these etiological agents by conventional laboratory methods can take up to 36 h or lead to false-negative results. Currently the isolation rate for cultivation of these microorganisms in public hospitals may reach only 30% [5]. The rapid diagnosis of these agents is important for patient management and for health surveillance actions to contain possible outbreaks. To decrease the time of diagnosis and increase the specificity and sensitivity of detection, several molecular methods have been developed based on the amplification of specific regions of bacterial DNA by PCR directly from clinical samples [5–8].

Real-time PCR (the TaqMan® system) is an upgrade of the conventional qualitative PCR (qPCR) method that was developed to quantify the DNA in the sample, but it can also be used to simply detect the presence of specific bacterial DNA, giving faster and more accurate results [9]. A variant of qPCR is called high-resolution melting (HRM). qPCR-HRM differs from qPCR-TaqMan in that it is a probe-free system; the reaction is carried out with a DNA-intercalating fluorescent dye which releases fluorescence when new dsDNA copies of the target are amplified [10,11]. After the PCR reaction, a melting curve is generated and a specific melting temperature (T_m) is achieved for each DNA sequence. The sensitivity of HRM is so high that it can detect T_m variations of less than 0.5°C in the presence of single-nucleotide polymorphisms (SNPs) [12]. Because no probes are included, HRM is easier and more cost-effective than probe-based genotyping analysis and, unlike conventional methods, it prevents carry-over contamination of PCR products. Detection and genotyping of bacterial pathogens with qPCR-HRM have been widely reported [10–15], but to our knowledge the method has not yet been applied for the detection of bacteria causing invasive diseases and meningitis. Because of its lower cost and ease, the qPCR-HRM protocol described here can be used in public laboratories for the identification of the three pathogens.

Materials & methods

Control strains

Reference strains were used as controls for species diagnosis. All reference strains are part of the bacteria collection (CBRVS) at our institution. The distribution of positive controls was as follows: *N. meningitidis* serogroups A, B, C, D and W135; *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 9V, 14, 19A, 19F, 33, 41, 51, 61; *H. influenza* a, b, c, d, e, f, NT and biogroup *aegyptius*. For negative controls, bacterial species frequently isolated from meningitis and sepsis cases were used. Table 1 lists all the control strains analyzed in the study.

Clinical samples & strains

A total of 394 clinical samples received from 2011 to date were tested by HRM. The sources of isolation were: cerebrospinal fluid with 152 (38.9%) samples and blood/sera with 186 (47.6%); 52 samples (13.3%) were of unknown source.

Negative clinical samples (cerebrospinal fluid, blood and sera) from suspected cases of invasive disease for the three pathogens after laboratory tests (latex agglutination, culture and microscopy) are routinely sent to our laboratory and submitted to culture and to end point PCR for pathogen detection.

We also analyzed a total of 176 strains isolated from clinical samples (*N. meningitidis*, n = 85; *H. influenzae*, n = 46; *S. pneumoniae*, n = 45) which are part of the CBRVS bacterial culture collection. The isolates were recovered from clinical samples after inoculation in chocolate agar (blood agar base with 5% sterile lysate defibrinated rabbit blood) and incubated at 37°C in a 5% CO_2 environment. Species confirmation was carried out by colony morphology, latex agglutination of specific antisera (Slidex® Meningitis kit; bioMérieux, Marcy-l'Étoile, France), Gram staining and optochin susceptibility (the latter only for *S. pneumoniae* determination). Each species was confirmed by species-specific conventional PCR tests using protocols previously reported [15–17]. These strains were preserved freeze-dried and incorporated into the CBRVS culture collection.

Primer design & PCR conditions

Species-specific primers were designed targeting exclusive genes for each pathogen with OligoArchitect™ software (Sigma-Aldrich, MO, USA) [18]. Template sequences were obtained from GenBank [19]. Table 2 lists primer sequences for qPCR-HRM. Genomic DNA extraction and purification from control strains, isolates and clinical samples was carried out using the DNeasy® Blood and Tissue extraction kit (Qiagen, MD, USA) according to manufacturer's instructions. Purified DNAs were kept at -20°C for repeated experiments. Two specific master mixes optimized with DNA-intercalating fluorescent dyes were used: Type-it® HRM PCR Kit (Qiagen) and MeltDoctor™ HRM Master Mix (Applied Biosystems, CA, USA). These two master mixes were used in two different real time PCR cyclers, respectively: the Qiagen Rotor-Gene Q 5plex Platform and Applied Biosystems QuantStudio™ 7 Pro real-time PCR system. Reactions were performed in 10-µl reaction mixtures containing 5 µl of Type-it HRM or MeltDoctor HRM master mix, 5 µM of each primer, ~20 ng/µl of genomic DNA and 2 µl of DNase- and RNase-free distilled water. PCR conditions were as follows: one cycle of 95°C for 5 min (Type-it HRM) or 10 min (MeltDoctor HRM), then 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 10 s. The HRM step for the dissociation curve and fluorescence reading was performed immediately after PCR cycling according to the instructions for each thermocycler. Reaction mixtures without DNA template were used as negative controls. Primer specificity was determined using DNA from control strains. Initially, specificity and sensitivity were tested by conventional PCR to confirm the amplification of the targets. After the conventional PCR, the amplifications were performed by qPCR-HRM to determine the minimum number of copies that the primers were able to detect in fg/ml.

Patent application

The diagnostic system proposed here has been approved for patent application in Brazil (no. BR 10.2018.003245.3), with pending patent applications in the USA, Canada, Europe and China (no. PCT/BR.2019/050049).

Results & discussion

Several PCR-based molecular methods for bacterial detection are currently available. Most of these methodologies are based on real-time PCR (TaqMan), a quantitative method that can also be used for qualitative purposes for DNA detection and is considered more sensitive and specific than end point PCR, showing a limit of detection of between one and ten DNA copies [6]. However, for the time being, DNA probes are still too expensive and the use of TaqMan methodology is not broadly applied to public laboratories, at least in developing countries. Moreover, the TaqMan method is not suitable for SNP detection because probes may hybridize equally to two DNA regions with slight differences depending on the SNP position. As a variation of the TaqMan system, HRM is based on the analysis of PCR products' melting profiles using saturating, dsDNA-binding fluorescent dyes to monitor the transition from unmelted to melted DNA at a specific temperature [5,14]. The HRM approach has been successfully used for genotyping and mutation scanning and is considered a fast, cost-effective, sensitive, specific and high-throughput SNP genotyping method [13,20–25]. In this study we used HRM for the detection of specific gene sequences of three human pathogens causing invasive diseases: *N. meningitidis, H. influenzae* and *S. pneumoniae*. The first step of HRM with the specific amplification of the target and fluorescence release would be sufficient for bacterial detection; however, because of the lack of specific probes tagged with different fluorophores, a multiplex approach for simultaneous detection of the pathogens would not be possible. The addition of a melting step showing specific melting profiles for each different

Table 1. Control strains used for qualitative PCR high-resolution melting analysis.

Bacterial species/serogroup or serotype	Source	Pathogen detected
Neisseria meningitidis A	ATCC 13077	1
N. meningitidis B	ATCC 13090	1
N. meningitidis C	ATCC 13102	1
N. meningitidis W135	ATCC 35559	1
N. meningitidis D	ATCC 13113	1
Haemophilus influenzae	ATCC 33391	2
H. influenzae aegyptius	ATCC 11116	2
H. influenzae NT	ATCC 49247	2
H. influenzae a	ATCC 9006	2
H. influenzae b	ATCC 33533	2
H. influenzae c	ATCC 9007	2
H. influenzae d	ATCC 9008	2
H. influenzae e	ATCC 8142	2
H. influenzae f	ATCC 9833	2
Streptococcus pneumoniae	ATCC 33400	3
S. pneumoniae 1	ATCC 27088	3
S. pneumoniae 14	ATCC 6314	3
S. pneumoniae 3	ATCC 6303	3
S. pneumoniae 33	ATCC 8333	3
S. pneumoniae 41	ATCC 10341	3
S. pneumoniae 51	ATCC 10351	3
S. pneumoniae 19F	ATCC 49619	3
S. pneumoniae 61	ATCC 10361	3
S. pneumoniae 19A	ATCC 700673	3
S. pneumoniae 14	ATCC 700672	3
S. pneumoniae 9V	ATCC 700671	3
S. pneumoniae 6B	ATCC 700670	3
S. pneumoniae 4	ATCC BAA-334	3
S. pneumoniae 6A	ATCC BAA-659	3
S. pneumoniae 5	ATCC BAA-341	3
N. lactamica	ATCC 23970	Neg
N. subflava	ATCC 11076	Neg
Haemophilus parasuis	ATCC 19417	Neg
H. parainfluenzae	ATCC 33392	Neg
Moraxella catarrhalis	ATCC 25238	Neg
S. agalactiae	ATCC 13813	Neg
S. pyogenes	ATCC 19615	Neg
Klebsiella pneumoniae	ATCC 13883	Neg
Listeria monocytogenes	ATCC 15313	Neg
Acinetobacter sp.	ATCC 14293	Neg
Escherichia coli	ATCC 11775	Neg
N. perflava	ATCC 11076	Neg
S. pyogenes	ATCC 12344	Neg

1 = *Neisseria meningitidis*; 2 = *Haemophilus influenzae*; 3 = *Streptococcus pneumoniae*.
ATCC: American Type Culture Collection; Neg: No amplification observed.

sequence allows the use of the method in a multiplexed approach. In this study, three specific melting patterns have been determined for each pathogen. These patterns were used for gene-specific identification of each species directly from bacterial isolates or from clinical samples with negative results after testing with conventional laboratory methods.

A total of 394 clinical samples received from 2011 to date were tested by HRM, of which 209 (53.6%) were positive for at least one of the three pathogens as follows: *N. meningitidis* with 176 (45.1%) positive samples, *S. pneumoniae* with 21 (5.4%) and *H. influenzae* with

Table 2. Primer sequences used and melting temperature for each target.

Gene target/species	Primer 5′–3′	Amplicon size	HRM range (±0.5°C)	
			RotorGene	QuantStudio 7
nspA	CAAGCTCTTTAGGTTCTG	137 bp	84.5	85.8
Neisseria meningitidis	GCTGTAAAGTTTGAAATCG			
P6	GAAGGTAATACTGATGAACG	134 bp	78.5	80
Haemophilus influenzae	TCACCGTAAGATACTGTG			
Ply	CCAAGTCTATCTCAAGTTG	86 bp	76.1	77
Streptococcus pneumoniae	CTACCTTGACTCCTTTTATC			

HRM: High-resolution melting.

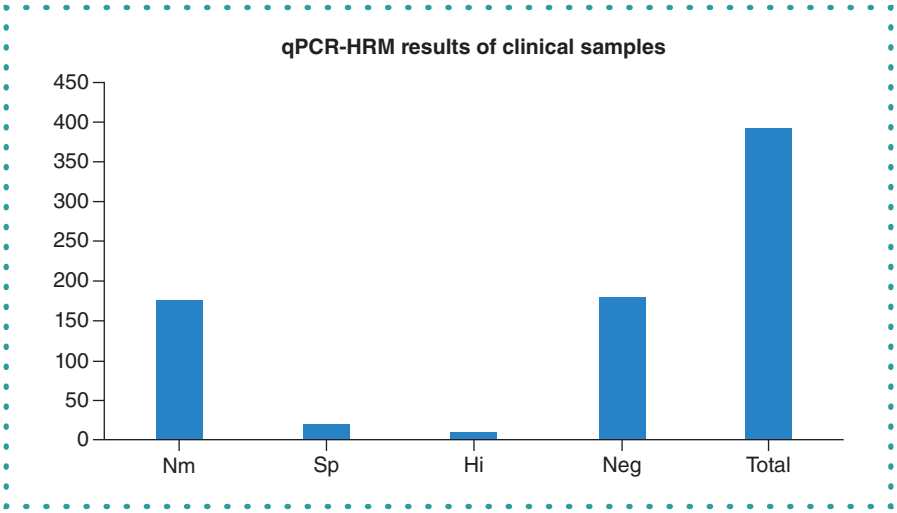

Figure 1. Results of clinical samples.
Hi: *Haemophilus influenzae*; Neg: Negative; Nm: *Neisseria meningitidis*; qPCR-HRM: qualitative PCR with high-resolution melting; Sp: *Streptococcus pneumoniae*.

12 (3%); there were 181 (46.4%) negative samples. Figure 2 shows the distribution of the samples with positive and negative results after qPCR-HRM analysis.

Our results shows that qPCR-HRM was sensitive and specific to accurately identify *N. meningitidis*, *S. pneumoniae* and *H. influenzae* from different sources (Figure 1). All clinical samples used in this study were submitted to the gold-standard method for identification of these pathogens, which is culture in specific media under specific conditions. Some isolates analyzed were recovered from these clinical samples after culture. From other samples, however, it was not possible to isolate an etiological agent. These samples were considered negative by conventional laboratory methods and have also been analyzed under qPCR-HRM.

Real-time monitoring of DNA amplification and a further melting step allow fast and accurate detection of the pathogens because the presence of target DNA is detected by fluorescence with no need for subjective visualization through agarose gel. We have established a specific and unique T_m for each pathogen which is able to determine the presence of genomic DNA from that species in clinical samples. The most important advantage of the HRM protocol described here compared with the TaqMan system is the overall cost. Using the same thermal cycler for qPCR-TaqMan, the cost of reagents is almost the same for end point PCR with no need for electrophoresis apparatus and reagents. TaqMan real-time PCR using probes can be considered as faster and as accurate as HRM, but the cost of three different probes for the simultaneous detection of each pathogen would greatly increase the overall cost of the assay. Compared with end point PCR, qPCR-TaqMan and qPCR-HRM show higher sensitivity and reduce turnaround time of the assay to ~2 h 30 min because there is no need for visualization of the amplicons by gel electrophoresis. According to our findings, all positive samples tested by PCR were confirmed by HRM, with 100% match of the species previously detected. Additionally, HRM was more sensitive than end point PCR to identify bacterial DNA: 31% of negative samples were confirmed as positive for one of the three pathogens by HRM.

To our knowledge, this is the first report of the application of qPCR-HRM for the simultaneous detection of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* from clinical samples using a single-step approach as described in this study. Because of its low cost and high sensitivity and specificity, the use of this method for diagnostic purposes by public laboratories could improve diagnosis of acute invasive bacterial disease, with clear benefit for patients and no additional costs.

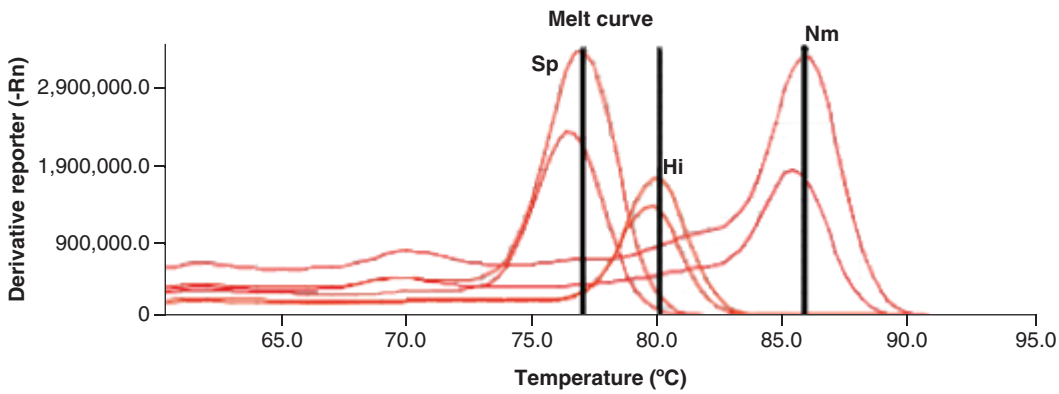

Figure 2. Melting curve showing melting temperature peaks for each target gene in the QuantStudio 7 thermocycler. Nm: 85.8°C, Hi: 80°C, Sp: 77°C. Hi: *Haemophilus influenzae*; Nm: *Neisseria meningitidis*; Sp: *Streptococcus pneumoniae*.

Conclusion

This is the first report of the use of qPCR-HRM for rapid and cost-effective diagnostic of bacterial meningitis. qPCR-HRM was used for simultaneous detection of the three most frequent bacterial agents of meningitis and invasive disease and was shown to be as sensitive and specific as the gold-standard test, qPCR-TaqMan. Additionally, the overall reagent costs of qPCR-HRM are about 20% those of the TaqMan system.

Future perspective

The protocol here presented may be used in public hospitals and laboratories as a sensitive and specific cost-effective new diagnostic tool. We believe that the distribution of this protocol in a great number of public laboratories in developing countries may improve the diagnosis of meningitis, with a clear benefit for all patients.

Executive summary

Background

- A rapid, sensitive and cost-effective molecular diagnostic method to detect bacterial meningitis was developed.

Materials & methods

- Qualitative PCR with high-resolution melting (qPCR-HRM) was used to detect the three most frequent and lethal bacterial etiological agents of meningitis.
- The protocol here proposed has been tested against isolated strains and clinical samples from patients with suspected meningitis, previously diagnosed as negative.
- The qPCR-HRM method is based on the specific melting temperature of each gene target, according to their different nucleotide sequences.

Results & discussion

- Clinical samples tested positive by conventional PCR were 100% confirmed by qPCR-HRM.
- Among negative samples, 31% tested positive by qPCR-HRM.

Conclusion

- qPCR-HRM proved to be a sensitive, specific and cost-effective tool for the diagnostic of bacterial meningitis.

Author contributions

I de Filippis: conceptualization, methodology investigation, funding acquisition, analysis of results, writing, review, editing, discussion of results and submission. A Carvalho de Azevedo: methodology, experiments supervision, analysis of results. I de Oliveira Lima: experimental procedures. N Lima Ramos: experimental procedures. C Ferreira de Andrade: experimental procedures. A de Almeida: review, discussion of results.

Acknowledgments

The authors are grateful to the Central Reference Laboratories (LACEN-PE, LACENRJ, LACEN-BA) and Hospital Municipal Lourenço Jorge-RJ for kindly providing strains and clinical samples. The authors are grateful to the sequencing core 'Plataforma Genômica de Sequenciamento de DNA/PDTIS-FIOCRUZ' and Instituto de Biologia Molecular do Paraná for the supply of reagents.

Financial & competing interests disclosure

This work was supported by PDTIS-FIOCRUZ (Proc. RID-31), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ (Proc. E-26/103.033/2012), Programa de Pós-Graduação em Vigilância Sanitária – PPGVS (INCQS/FIOCRUZ), Programa de Inovação Tecnológica (FIOCRUZ-INOVATEC), Programa Fiocruz de Fomento à Inovação, (INOVA-Fiocruz), *InovaBio (Bio-Manguinhos*/Fiocruz) and INCQS/FIOCRUZ. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil, Finance code 001. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study was approved by the Ethics Committee for Scientific Research CEP-IOC/FIOCRUZ, (CAAE 52531715.0.0000.5248).

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Edmond K, Clark A, Korczak VS, Sanderson C, Griffiths UK, Rudan I. Global and regional risk of disabling sequelae from bacterial meningitis: a systematic review and meta-analysis. *Lancet. Infect. Dis.* 10(5), 317–328 (2010).
2. Rouphael NG, Stephens DS. *Neisseria meningitidis*: biology, microbiology, and epidemiology. *Meth. Mol. Biol.* 799, 1–20 (2012).
3. World Health Organization. The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries and risk factors in 1990 and projected to 2020. In: *The Global Burden of Disease.* Harvard University Press, MA, USA (2001).
4. World Health Organization. Global literature review of Haemophilus *influenzae* type b and *Streptococcus* pneumonia invasive disease among children less than five years of age: 1980–2005 [WHO/IVB/09.02]. http://whqlibdoc.who.int/hq/2009/WHO_IVB_09.02_eng.pdf
5. de Filippis I, de Andrade CF, Caldeira N, de Azevedo AC, de Almeida AE. Comparison of PCR-based methods for the simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in clinical samples. *Braz. J. Infect. Dis.* 20(4), 335–341 (2016).
6. Ouattara M, Whaley MJ, Jenkins LT *et al.* Triplex real-time PCR assay for the detection of *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* directly from clinical specimens without extraction of DNA. *Diagn. Microbiol. Infect. Dis.* 93(3), 188–190 (2019).
7. Sacchi CT, Fukasawa LO, Gonçalves MG *et al.* Incorporation of real-time PCR into routine public health surveillance of culture negative bacterial meningitis in São Paulo, Brazil. *PLOS ONE* 6(6), e20675 (2011).
8. Meyler KL, Meehan M, Bennett D, Cunney R, Cafferkey M. Development of a diagnostic real-time polymerase chain reaction assay for the detection of invasive *Haemophilus influenzae* in clinical samples. *Diagn. Microbiol. Infect. Dis.* 74(4), 356–362 (2012).
9. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J. Clin. Microbiol.* 39, 1553–1558 (2001).

• **First study using qualitative PCR (qPCR) TaqMan for diagnostic of the three meningitis agents.**

10. Douarre PE, Cashman W, Buckley J, Coffey A, O'Mahony JM. High resolution melting PCR to differentiate *Mycobacterium avium* subsp. Paratuberculosis 'cattle type' and 'sheep type'. *J. Microbiol. Meth.* 88, 172–174 (2012).

•• **Demonstrates the use of qPCR–high-resolution melting (HRM) for genotyping.**

11. Zeinzinger J, Pietzka AT, Stoger A *et al.* One-step triplex high-resolution melting analysis for rapid identification and simultaneous subtyping of frequently isolated *Salmonella* serovars. *Appl. Environ. Microbiol.* 78, 3352–3360 (2012).

•• **Demonstrates the usefulness of qPCR-HRM for diagnostic genotyping.**

12. Gori A, Cerboneschi M, Tegli S. High-resolution melting analysis as a powerful tool to discriminate and genotype *Pseudomonas savastanoi* pathovars and strains. *PLOS ONE* 7, e30199 (2012).

•• **Explains the molecular basis of qPCR-HRM.**

13. Cheng JC, Huang CL, Lin CC *et al.* Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. *Clin. Chem.* 52(11), 1997–2004 (2006).
14. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* 8(6), 597–608 (2007).

•• **Demonstrates the use of qPCR-HRM for diagnosis.**

15. de Souza Santos DR, do Carmo CN, de Filippis I. High-resolution melting (HRM) for rapid MLST analysis of *Neisseria meningitidis*. *J. Microbiol. Methods* 200, 106557 (2022).

•• **Demonstrates the use of qPCR-HRM for genotyping.**

16. van Ketel RJ, de Wever B, van Alphen L. Detection ofHaemophilus influenzaein cerebrospinal fluids by polymerase chain reaction DNA amplification. J. Med. Microbiol. 33(4), 271–276 (1990).
17. de Filippis I, do Nascimento CR, Clementino MB *et al.* Rapid detection ofNeisseria meningitidisin cerebrospinal fluid by one-step polymerase chain reaction of the nspA gene. Diagn. Microbiol. Infect. Dis. 51(2), 85–90(2005).
18. Sigma-Aldrich. Oligo Architect. www.oligoarchitect.com/
19. NCBI. GenBank. www.ncbi.nlm.nih.gov/genbank/
20. 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.* 49(3), 396–406 (2003).
21. Cho MH, Ciulla D, Klanderman BJ, Raby BA, Silverman EK. High-resolution melting curve analysis of genomic and whole-genome amplified DNA. *Clin. Chem.* 54(12), 2055–2058 (2008).
22. Druml B, Cichna-Markl M. High resolution melting (HRM) analysis of DNA – its role and potential in food analysis. *Food Chem.* 1(158), 245–254 (2014).
23. Cai XQ, Yu HQ, Ruan ZX *et al.* Rapid detection and simultaneous genotyping of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) in powdered infant formula using real-time PCR and high resolution melting (HRM) analysis. *PLOS ONE* 8(6), e67082 (2013).
24. Tamburro M, Ripabelli G. 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. *Ann. Ig.* 29(4), 293–316 (2017).

•• **Demonstrates the cost–effectiveness of qPCR-HRM.**

25. Tamburro M, Sammarco ML, Ripabelli G. High resolution melting analysis for the characterization of lineage II *Listeria monocytogenes* serovars 1/2a and 1/2c based on single nucleotide polymorphisms identification within the Listeria Pathogenicity Island-1 and inlAB operon: a novel approach for epidemiological surveillance. *J. Appl. Microbiol.* 125(6), 1920–1937 (2018).

•• **Demonstrates the use of qPCR-HRM for epidemiological surveillance.**