Comparative Immunology, Microbiology and Infectious Diseases 67 (2019) 101364

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Review

High-resolution melt curve analysis: A real-time based multipurpose approach for diagnosis and epidemiological investigations of parasitic infections

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ARTICLE INFO

Keywords:
Molecular diagnosis
HRM real-time PCR
Parasitic infection
Multipurpose method
Systematic review

ABSTRACT

Background: Real-time PCR coupled with high resolution melting curve analysis is a practical technique that could be employed in multipurpose studies. During the recent decade, this technique has been practiced for different targets, worldwide.
Methods: In the current study three major database centers consisted of PubMed, Scopus and Web of Science were searched until Aug 2019 for applications of HRM real-time PCR in parasitology studies using terms: "Parasite" AND "HRM real-time PCR" OR "High Resolution Melting curve analysis" OR "Real-time PCR", "Protozoan parasites" AND "HRM real-time PCR" OR "High Resolution Melting curve analysis" OR "Real-time PCR", "Helminth" AND "HRM real-time PCR" OR "High Resolution Melting curve analysis" OR "Real-time PCR".
Results: Totally, 83 papers met our criteria and were included in our study. This method was more frequently used for protozoan parasites (52/83; 62.65%), while lower (31/83; 37.35%) studies were incorporated on helminths parasites. Furthermore, *Plasmodium* spp., and *Leishmania* spp., were the most prevalent protozoan parasites, and *Taenia* spp., and filers were the most frequent helminths that were studied by HRM real-time PCR.
Conclusion: HRM real-time PCR is a sensitive, flexible and cost-effective method that could be used for multipurpose studies.

1. Background

Despite improvement of standards of living in most of countries/regions, there are several reports from almost all countries showing the occurrence of parasitic infections in both immunocompetent and immunocompromised subjects [1–3]. It is estimated that approximately 25% of world's population have experienced infection with at least one parasite during their life [4–6]. However, the prevalence of parasitic infections is mostly related to hygiene conditions of a region [7]. Although significant clinical manifestations are not usually reported from most of parasite-infected immunocompetent subjects, immune disorders increase the risk of complications due to parasitic infections, particularly opportunistic parasites [8–10]. On the other hand, some of parasitic protozoans such as *Entamoeba histolytica* [11–13], *Giardia lambelia* [14,15], *Negleria* spp. [16,17], human-infecting species of *Leishmania* [18] and *Plasmodium* [19], together with some helminthic parasites, are known as true pathogens [20–23]. Therefore, rapid and accurate diagnosis of parasites plays crucial role in early detection, treatment process and prognosis of affected patients.

For years, traditional methods, particularly microscopical examination, have been employed as a routine test for detection of parasites. Although microscopical examination is the main employed technique in most of regions with traditional facilities, DNA-based methods for clinical diagnosis and molecular epidemiology of parasitic

Abbreviations: HRM, high resolution melting; PCR, polymerase chain reaction; EPHPP, effective public health practice project quality assessment tool; PRISMA, preferred reporting items for systematic reviews and meta-analyses; SNPs, single nucleotide polymorphisms; IBS, inflammatory bowel syndrome

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https://doi.org/10.1016/j.cimid.2019.101364
Received 19 June 2019; Received in revised form 23 September 2019; Accepted 24 September 2019

diseases have developed due to their higher sensitivity and specificity [24,25]. Real-time PCR coupled with high resolution melting (HRM) curve analysis is a molecular method, which was firstly described in a complex of studies conducted by Carl Wittwer's team [26]. This technique acts based on differences in nucleotide content of targeted fragment of a gene that leads to difference in the melting temperature (Tm) of the amplified fragment [26,27]. This technique was frequently practiced for detection, genotyping and studies on drug resistance in parasites [28–31]. Recently, quantitative HRM real-time PCR was developed for simultaneous detection and genotyping of parasites [32]. In the current study, we systematically gathered all papers in the field of parasitology that used HRM real-time PCR for detection, quantitation, genotyping as well as characterization of the genes responsible for drug resistance in parasites.

2. Materials and methods

2.1. Search strategy

In order to gather papers that used HRM real-time PCR in the field of parasitology, available English electronic libraries including: PubMed, Scopus and Web of Science were searched until Aug 2019 with keywords: "Parasite" AND "HRM real-time PCR" OR "High Resolution Melting curve analysis" OR "Real-time PCR", "Protozoan parasites" AND "HRM real-time PCR" OR "High Resolution Melting curve analysis" OR "Real-time PCR", "Helminth" AND "HRM real-time PCR" OR "High Resolution Melting curve analysis" OR "Real-time PCR".

2.2. Eligibility of studies

All papers were independently evaluated by two authors (H M R and H M). Accordingly, papers that met the following criteria consist of a) providing sufficient data about the designing test, 2) those studies that were performed by specialized scientists in the field of parasitology, 3) providing reliable data such as Tm, primers and relevant master mix, and 4) providing clear data about the place of study, were included in the current study.

2.3. Quality assessment and data extraction

In order to evaluate the quality of selected papers, all of them were read and investigated by two mentioned parasitologists. Therefore, paper information such as 1) relevance of title and text of the papers and 2) methods that were used for detection of parasites were considered. In addition, included papers were scored based on Effective Public Health Practice Project Quality Assessment Tool (EPHPP) [33] in order to assess the risk of bias.

Finally, all selected papers that met our criteria were included in Excel (Microsoft office, 2016) according to the name of the first author, studied parasites, place of study and year of publication.

3. Results

Overall, 312 studies were identified based on searched keywords throughout the databases. Accordingly, 83 papers met our criteria based on PRISMA 2009 Flow Diagram (Fig. 1, Supplementary table 1) and were included in the current study. Concerning the searches in well-known databases, the first studies that employed HRM real-time PCR on the parasites were conducted in 2009 (Fig. 2). However, during the years, real-time PCR coupled with HRM was more frequently used for protozoan parasites (52/83; 62.65%), while (31/83; 37.35%) of studies were incorporated on helminths parasites. Furthermore, *Plasmodium* spp., and *Leishmania* spp., (Fig. 3) were the most prevalent protozoan parasites and *Taenia* spp., and filers (Fig. 4) were the most frequent helminths that were studied by HRM real-time PCR.

Searching among the selected papers showed four main proposes including: a) only species/genotype identification, b) determination of mutations involved in drug resistance, c) only detection of parasites, and d) simultaneous detection and species/genotype identification of parasites (Fig. 5). All data including primers, range of Tm, relevant master mix, targeted genes and the scope of studies were extracted (supplementary table 2).

4. Discussion

4.1. A history of techniques for detection of parasites

Despite impressive improvements in quality of life and hygiene conditions in many countries during the past decades, parasitic infections still place among the list of infectious agents that are frequently reported from particularly, undeveloped and developing regions [34,35]. Apart from this fact, together with increasing our knowledge about the immunological disorders as well as HIV/AIDS pandemic, some parasites have emerged/reemerged in the recent decades [9,36–39]. On the other hand, concerning the need of world's population to food, the number of industries related to animal products have explosively increased and thus, the risk of zoonotic transmission of parasites from animal origins have elevated [4,40].

Although observing parasites in clinical samples is considered golden standard, diagnosis of parasite using conventional methods such as light microscopy might not be useful, particularly in patients with low number of parasite. Indeed, microscopical examination of clinical samples, particularly stool specimen, is time-consuming and needs well-trained technician [24]. Numerous immunological assays have been introduced and experienced to help clinical laboratories for detection of some parasitic infections [41,42]. However, one of the main limitations of immunological tests is diagnosis of parasitic infections in patients with immune disorders. Furthermore, cross-reaction between parasites have limited application of serological tests in most of parasitic infections [43,44].

During the recent decades, limitations of conventional techniques have led to development of DNA-based technique for clinical diagnosis and molecular epidemiology of parasitic diseases [45,46]. The most important advantages of molecular tests in comparison with previously developed conventional techniques are a) higher sensitivity and specificity of molecular tests, b) reducing the final costs of diagnostic tests and c) decreasing the time of tests, particularly those infections with acute progress [47]. However, in comparison with conventional microscopical and serological tests, molecular techniques are not only able to determine multiple species/genotypes/subtypes of parasites, but also can provide useful information about quantitation of a parasite in an infection [45,46].

4.2. Real-time PCR coupled with HRM is a suitable method for simultaneous detection and characterization

Despite of many molecular techniques, real-time PCR is widely suggested as a quantitative method for rapid detection of parasites [48,49]. Real-time PCR coupled with HRM is a multipurpose method that have been employed for detection, species identification, genotyping and detection of single nucleotide polymorphisms (SNPs) [50,51] responsible for drug resistance, among broad range of parasites.

From the mid-2000′s that real-time PCR coupled with HRM was introduced and developed for different clinical applications [27], several studies employed this assay in the field of parasitology [29,32,52–56]. At the beginning, detection and species identification of parasites were the main targets in most of studies. Pangasa et al., practiced HRM real-time PCR to detect *Cryptosporidium* and its species in human patients. Accordingly, they could successfully differentiate *C. hominis*, *C. meleagridis* and *C. parvum* in stool samples [52]. This method was then employed by Hussein et al., for detection and analysis of genetic diversity of *Dientamoeba fragilis* among inflammatory bowel

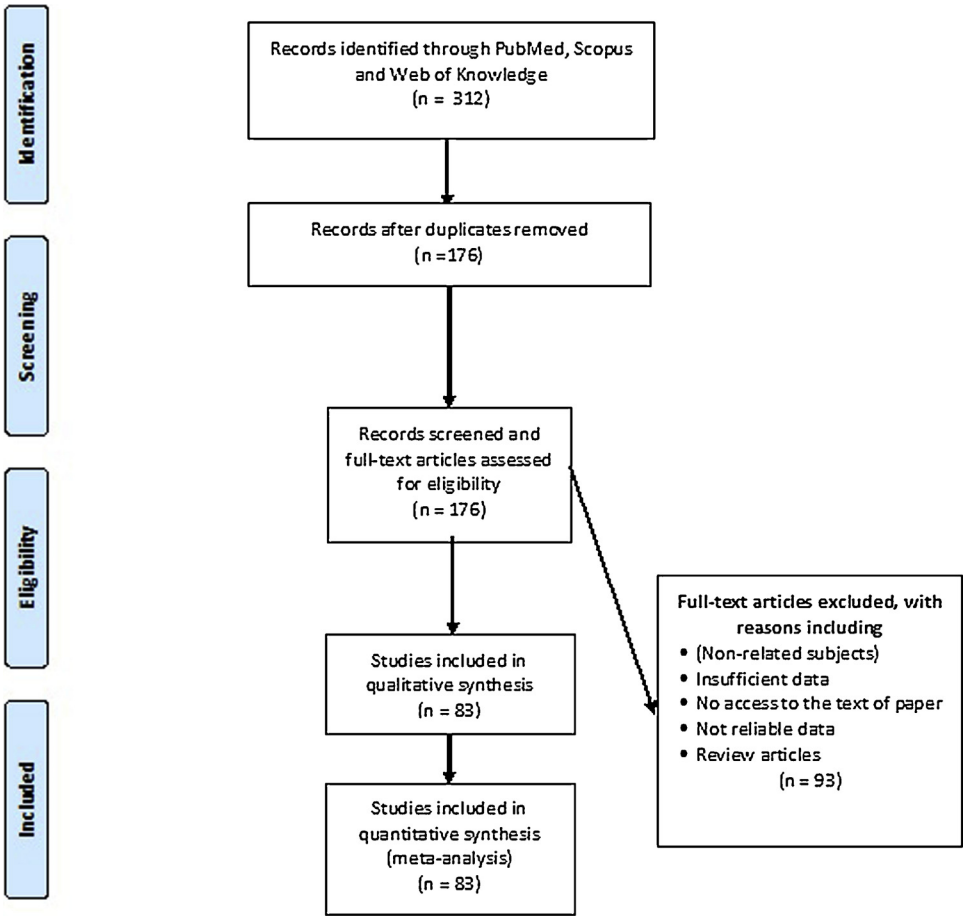

Fig. 1. PRISMA 2009 flow diagram shows the number of investigated and analyzed papers.

syndrome (IBS) patients [57]. During the years, concerning the high sensitivity of real-time approach and capability of real-time PCR coupled with HRM for simultaneous detection and species/genotype identification, this technique was frequently exploited for detection and characterization of parasites in clinical samples. In a study conducted by Kipanga et al., both nested HRM real-time PCR and direct HRM real-time PCR were shown to be able to detect low parasitaemia samples, while they were negative for *Plasmodium*, microscopically [58].

In addition, applying HRM real-time PCR for species identification and genotyping of parasites provides useful information about the origin of an infection, particularly in the case of zoonotic parasites. Many studies applied this method for description of zoonotic

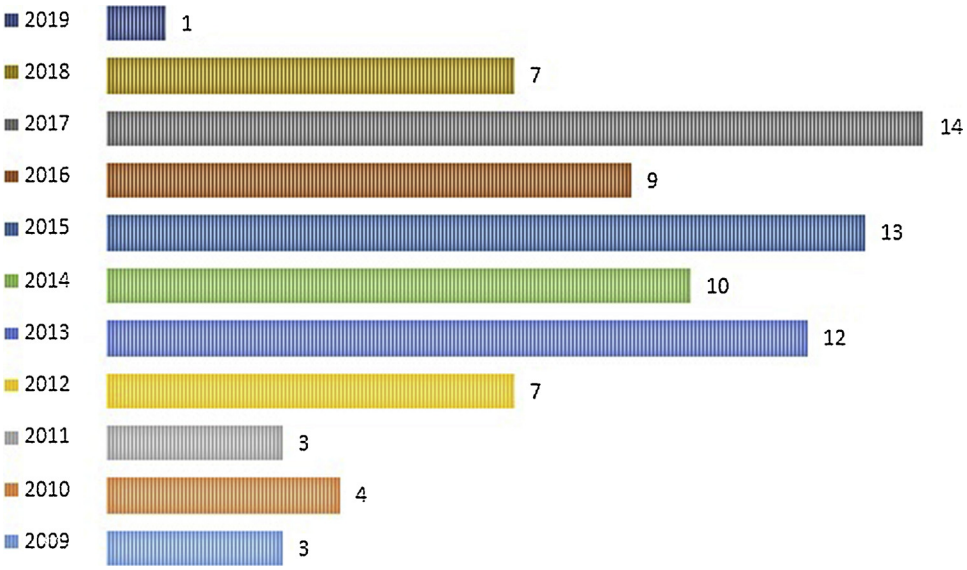

Fig. 2. This figure represents the number of studies from 2009 to Aug 2019, which were conducted on medical and veterinary important parasites. The figure shows that a growing number of studies has been performed from 2012.

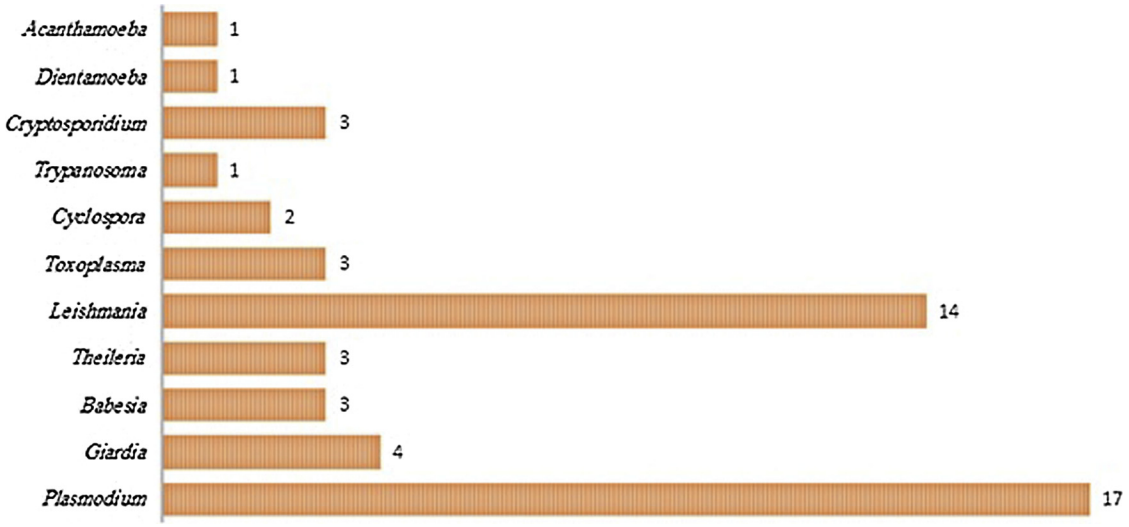

Fig. 3. This figure shows studies performed on protozoan parasites based on genus. Accordingly, most of studies were designed for detection, species identification as well as characterization of drug resistant for *Plasmodium* spp., and *Leishmania* spp.

assemblage of *Giardia* [59,60] as well as genotypes of *Echinococcus granulosus* [61]. Indeed, several studies employed HRM real-time PCR to characterize pathogenic species/genotypes of parasites. Based on the results of our analysis, most of researches around the world (90.36%; 75/83) used this method for both/either detection and/or species/genotype identification. Regarding the application of HRM real-time PCR for rapid detection and species identification of *Plasmodium* spp., this method seems to be able to overcome the limitations of conventional techniques. The main reason for this application of HRM real-time PCR comes from the ability of this method for rapid detection and characterization of tested parasites in clinical samples [58]. However, it seems that unpredicted mutations across the targeted fragment can lead to misinterpretation of a species or genotype and have to considered as disadvantage of HRM real-time PCR.

4.3. HRM real-time PCR for detection of drug resistance

Apart from genotyping, SNP detection throughout the genes that are responsible for drug resistance has been reported an interesting capacity of HRM real-time PCR [28,62,63]. During the past decade, many studies established HRM real-time PCR for detection of mutations, which are related to drug resistance, particularly in *Plasmodium* species. From the study conducted by Andriantsoanirina et al., who developed HRM real-time PCR for rapid detection of four genes associated with drug resistance in *Plasmodium* species [63], this technique was frequently employed in endemic regions for this parasite. However, it seems that this technique could be a rapid diagnostic test that can apply for multipurpose studies as well as clinical practices.

Overall, focusing on HRM real-time PCR shows that although many studies have been performed on filaria, there is no focus for detection of a particular helminth. In contrast to helminths, more than 30% of studies that employed HRM real-time PCR on protozoans, were conducted on *Plasmodium* spp., and *Leishmania* spp. However, this technique has some limitations including: a) presence of unpredicted mutation throughout the targeted fragments that leads to change in expected temperature range and b) lack of availability for the HRM real-time PCR instrument in clinical laboratories particularly in developing regions [26].

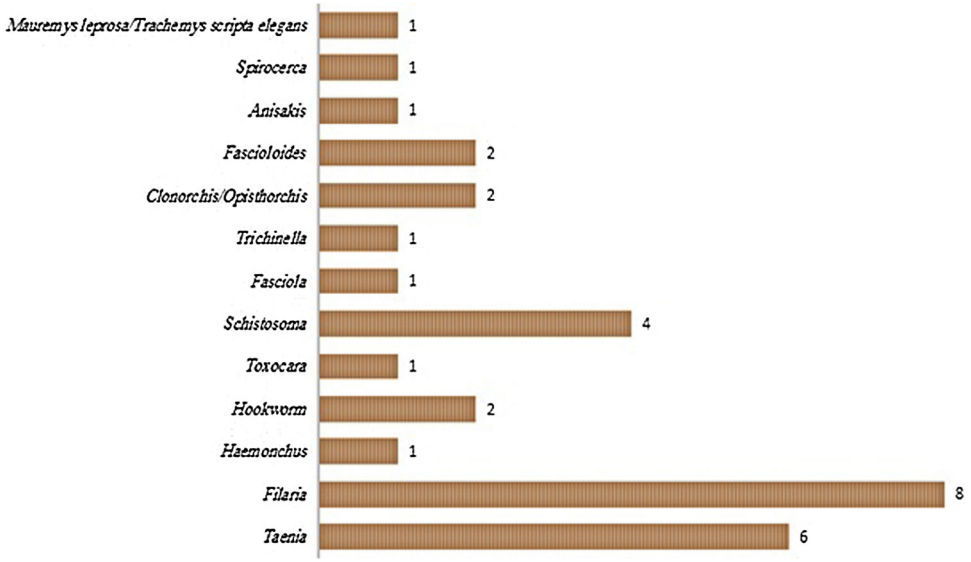

Fig. 4. This figure shows HRM real-time PCR, which were performed on helminth parasites based on the genus. The most number of studies which were designed for veterinary with zoonotic important helminth.

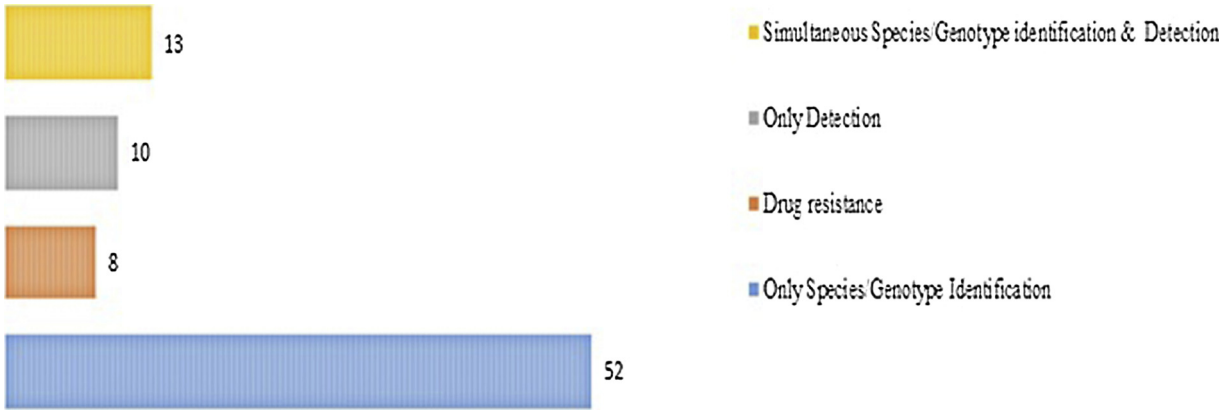

Fig. 5. An overview of purposes of studies performed from 2009 to Aug 2019. This figure shows that most of studies have focused on species identification and genotyping of parasites. It seems that since HRM real-time PCR for drug resistant was only employed for malaria and leishmaniasis, the number of studies in this field has been lower than others.

5. Conclusion

Taken together, HRM real-time PCR is a rapid, cost effective technique that could be used for multipurpose studies. Although due to some limitations, application of this technique has not been pervasive especially for clinical practice, some criteria such as high sensitivity, flexibility and cost effectiveness of this method make it a practical technique for screening of infectious diseases, particularly in endemic areas. In addition, it seems that capability of HRM real-time PCR for simultaneous detection and genotyping together with determination of burden of infection and presence of resistance genes, particularly in malaria infection, can provide plenty of information in clinical and epidemiological studies.

Author contributions

Conceived and designed the experiments: HMR HM MAP. Data gathering and testing eligibility of studies: HMR HM. Writing the manuscript: HM HMR. Reviewing and editing the manuscript: MAP AY MRZ.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Consent for publication

None.

Ethics approval and consent to participate

Not applicable.

Funding

This study was financially supported by Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant no. 970).

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to thank all the staffs of the Foodborne and Waterborne Diseases Research Center for their collaboration.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101364.

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