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Molecular detection of *Trichostrongylus* species through PCR followed by high resolution melt analysis of ITS-2 rDNA sequences

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ABSTRACT

Polymerase chain reaction followed by high resolution melting (PCR-HRM) analysis is a simple, rapid and accurate method for molecular detection of various nematode species. The objective of the present study was, for the first time, to develop a PCR-HRM assay for the detection of various animal *Trichostrongylus* spp. A pair of primers targeting the ITS-2 rDNA region of the *Trichostrongylus* spp. was designed for the development of the HRM assay. DNA samples were extracted from 30 adult worms of *Trichostrongylus* spp., the ITS-2-rDNA region was amplified using PCR, and the resultant products were sequenced and characterized. Afterwards, the PCR-HRM analysis was conducted to detect and discriminate *Trichostrongylus* spp. Molecular sequence analysis revealed that 24, 4, and 1 of the samples were *T. colubriformis*, *T. vitrinus* and *T. capricola*, respectively. Results from PCR-HRM indicated that complete agreement was relatively found between speciation by HRM analysis and DNA sequencing for the detection of *Trichostrongylus* species. The PCR-HRM analysis method developed in the present study is fast and low-cost; the method can be comparable with other molecular detection techniques, representing a reliable tool for the identification of various species within the *Trichostrongylus* genus.

1. Introduction

Intestinal parasitic nematodes are a major impediment to health and production in livestock industries worldwide. These nematodes are considered to be a major cause of economic losses, leading to decreased meat, wool, and milk production, as well as weight loss and increased mortality rates of livestock [1–3]. Among these nematodes, *Trichostrongylus* spp. were demonstrated to be highly prevalent and pathogenic in domesticated and wild herbivores. *Trichostrongylus* spp. are the major health challenges in the tropical regions, which are ubiquitous among herbivores. On the other hand, *Trichostrongylus* spp. occasionally infect humans, especially those live in the Middle East and Asia [4]. Epidemiological studies showed a worldwide distribution of *Trichostrongylus* infections in humans, having the highest prevalence rates in individuals from regions with poor sanitary conditions and rural areas, or in farmers/herders. Humans infected with the parasite present with stomachaches, abdominal bloating, diarrhea, and eosinophilia [5]. Out of more than 30 species infecting mammals, only 10 species are able to infect humans [6]. Seven species of Trichostrongylus have been reported in Iran, among which T.colubriformis was identified more frequently in human beings [7]. Therefore, more comprehensive parasitological and epidemiological studies are required to help practical prevention and control of this parasitic zoonosis.

To design effective parasite control strategies, there is a need for unequivocal detection and characterization of *Trichostrongylus* spp. for a better understanding of the epidemiology, anthelmintic treatment efficacy, and drug resistance. Microscopic examination of stool specimens for parasite eggs is currently a routine laboratory method for the detection of *Trichostrongylus*. However, microscopic examination faces a variety of critical challenges, including difficulty to identify different species due to similarity among nematode species, being time consuming, insensitive and nonspecific, as well as requiring some expertise and experience [8]. Therefore, it is extremely important to develop highly sensitive and reliable diagnostic methods capable of accurately identifying helminth eggs, which in turn help control parasitic infections among humans.

A great number of studies have highlighted the important role of molecular techniques to detect and discriminate various parasite species [9,10]. The second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (rDNA) was demonstrated to be a reliable genetic marker for the detection of a broad range of nematodes, ranging from the order Strongylidae to species [11]. In addition, the 18 S rRNA gene

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contains alternating regions of sequence conservation and heterogeneity. The conserved regions are frequently used for phylogenetic analysis of higher taxonomic orders (for example; phylum, family, and genus), whereas sequence diversity regions are of great importance for the characterization of isolates at genus or species levels. Indeed, isolates showing sequence identity more than 97 % in the 18S rRNA gene are generally considered as the identical species. The rRNA ITS-1 and ITS-2 gene regions, when compared with the rRNA genes, are generally more variable, representing valuable targets for speciation and identification. The identity of the testing organism is defined by its ITS sequence similarity (%) to the type strain or control isolates: species, ≥ 99 %; genus, 93–99 %; and inconclusive, ≤93 % [12].

PCR followed by sequencing can be an alternative molecular method to detect *Trichostrongylus* genus which is increasingly used for species confirmation [13,14]. PCR-sequencing, when compared with the conventional microscopic method, is more sensitive and is becoming increasingly available [15,16]. However, direct DNA sequencing is time-consuming and expensive for the widespread use of this technology [17].

Polymerase chain reaction followed by high resolution melting (PCR-HRM) analysis is an alternative molecular technique developed to screen variations in DNA sequences [18]. In this method, a fluorescent dye capable of binding to double-stranded DNA is added to amplicons resulting from the PCR reaction. When temperature increases, the double-stranded DNA is dissociated into single strands, leading to a decrease in fluorescence intensity that is measured by a detector. Variations in DNA sequences produce alterations in the melting characteristics of DNA amplicons, leading to differences in melting curve shapes of different DNA sequences. The application of this technique is less time-consuming, simpler, and more sensitive than previous techniques [19,20].

Quantitative real-time PCR (qPCR) assays provide a highly sensitive tool to diagnose and quantify parasitic infections. In addition, qPCR followed by high-resolution melt analysis (HRM) is a useful tool for the specific identification of DNA amplicons based on their melting temperatures, thereby facilitating fast diagnosis. A number of successful PCR-HRM analyses were previously used to distinguish a variety of parasite species in different genera, such as *Cryptosporidium* [21], *Giardia* [22,23], *Leishmania* [24], *Naegleria* [25], *Plasmodium* [26,27], *Schistosoma* [28,29], *Trypanosoma* [30], *Ancylostoma* [31], *Brugia* [32,33] and *Fasciola* [34], as well as strains of *Echinococcus granulosus* [35], and *Spirocerca lupi* [36]. To the best of our knowledge, PCR-HRM has not been used for the identification of *Trichostrongylus* spp. In the present study, we exploited PCR-HRM, employing ITS-2 as a genetic marker, for rapid differential diagnosis of various species of *Trichostrongylus* genus infecting sheep.

2. Materials and methods

2.1. Ethical statement

The study was approved by the Ethics Committee of Kashan University of Medical Sciences, Iran (Approval ID: IR.KAUMS.MEDNT.REC.1396.112, 2017).

2.2. Parasite preparation and microscopic detection

Thirty adult fresh *Trichostrongylus* samples isolated from 70 naturally-infected sheep were collected at slaughterhouse in Isfahan Province, Iran, during 2018. All the specimens were first examined using the 10x objective of a compound microscope for the identification of *Trichostrongylus* spp. based on standard keys [37,38]. The isolated worms were preserved in absolute ethanol and then transferred to the Parasitology Lab, for further study. The samples were extensively washed in PBS for three times to remove ethanol, and then stored at −20 °C for DNA extraction.

2.3. DNA extraction

Before extraction, adult worms were treated with lysis buffer, frozen in liquid nitrogen at −196 °C, and then thawed in hot water bath at 70–80 °C for three times (10 min for each step). The freeze & thaw method facilitates cell membrane breakage and the release of DNA for further experimentation. The genomic DNA of *Trichostrongylus* spp. was extracted from each fresh adult worm using AccuPrep tissue DNA Extraction Kit (Bioneer, Korea, K-3032) according to manufacturer's instruction. DNA quantity was determined using an Epoch™ Microplate Spectrophotometer (Biotek, USA). Finally, DNA from each sample was stored at −20 °C until the next step.

2.4. Primer design

Primers were designed from the ITS-2 region of the eight species of *Trichostrongylus* ribosomal DNA because the region contains both highly conserved and variable regions. ITS-2 sequences of *T. axei* (MH481571), *T. capricola* (JF276022), *T. colubriformis* (MH481550), *T. longispicularis* (KY355070), *T. probolurus* (JQ925867), *T. retortaefoemis* (KC521412), *T. rugatus* (KC521396), and *T. vitrinus* (JF680986) were obtained from NCBI GenBank, followed by multiple sequence alignment using CLC Main Workbench 5 (Toolbox: Create Alignment). After identification of the specific and conserved regions in the ITS-2 region for each *Trichostrongylus* spp., a pair of primers with degeneracy (Forward: TGTGATRATTCCCATTTYAGTH, and Reverse: YAAACAGTRACAACACCTTCACA) was designed for the HRM analysis. Primer-BLAST analysis was performed to check the specificity of the primer pair. Temperature suitable for primer annealing was determined by CLC Main Workbench 5 (Toolbox: Analyze Primer Properties). To determine the optimal primer annealing temperature for empirical specificity of PCR, temperature-gradient PCR was conducted using a LightCycler 96 system (Roche Diagnostics, Germany) at a final volume of 50 ul, containing 25 ul Taq 2x master mix (Ampliqon), 0.2 u M of each primer and 30 ng of genomic DNA. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 55 ± 5 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 30 min. Following gradient conventional PCR, electrophoresis was carried out for the PCR products on a 1 % agarose gel (w/v). Gel was stained with EchoRed (BioBasic, Canada) and visualized under the UV light by Gel Documentation System (SABZ Biomedicals, Iran).

2.5. PCR-HRM analysis

All PCR-HRM reactions were performed with 4 u L of 5x HOT FIREPol® EvaGreen® HRM Mix (Solis BioDyne, Estonia), 200 nM of each primer and 60 ng of DNA in a total volume of 20 u L, according to the manufacturer's protocol. A LightCycler® 96 system (Roche Diagnostics, Germany) was used for amplification, and the cycling conditions were as follows: for PCR, initial activation at 95 °C for 12 min; followed by 45 cycles of 95 °C for 15 s, 52 °C for 20 s and 72 °C for 20 s; and for HRM a dissociation cycle consisting of 95 °C for 60 s, 40 °C for 60 s, 65 °C for 1 s, and 97 °C for 1 s. Each PCR run contained a negative control (no template). Data were acquired and analyzed using LightCycler® 96 SW1.1. All curves were analyzed following normalization, temperature shifting, and the inspection of different plots. All amplified products were sequenced (Bioneer, Korea) and the resulting data were analyzed to ensure the homology of ITS-2 regions using BLASTn. Sequence analysis was performed using CLC Main Workbench 5 software.

3. Results

The *Trichostrongylus* genus was characterized in 70 samples using morphological examinations, and molecular studies were conducted on 30 samples (numbered as 1–30). One pair of primers was designed using ITS-2 rDNA sequences of *Trichostrongylus* spp. (Fig. 1A). The *in*

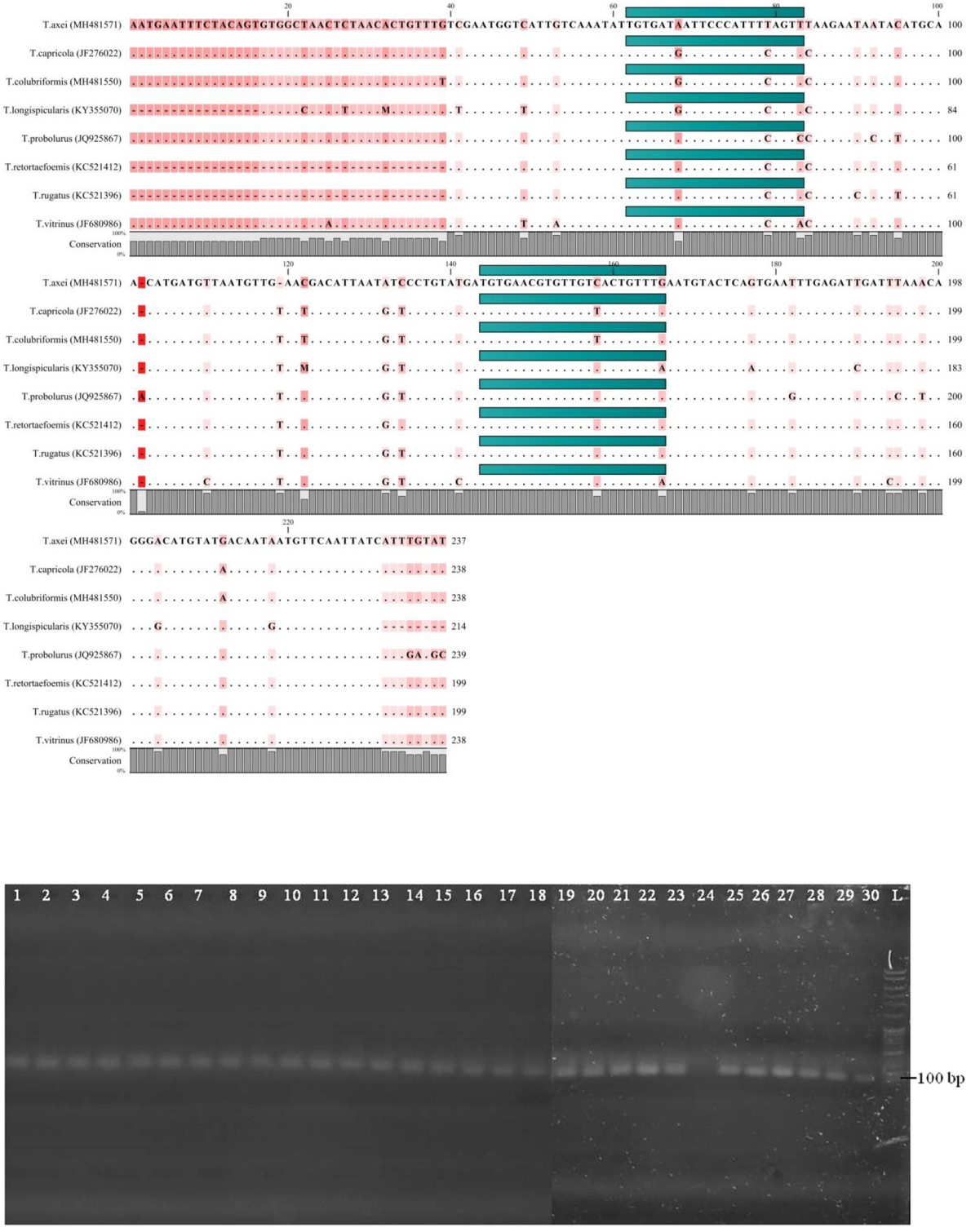

Fig. 1. (A) The multiple sequence alignment for consensus ITS-2 sequences of eight Trichostrongylus spp. The sequences were aligned using CLC Main Workbench 5. The green boxes represent the common forward and reverse of the eight Trichostrongylus spp. (B) Electrophoresis bands resulting from amplification of ITS-2 rDNA regions using the common primer pair for the eight *Trichostrongylus* spp.

silico study of the pair primer, designed to amplify the target sequence of the ITS-2 rDNA region, was found to be specific to *T. axei*, *T. capricola*, *T. colubriformis*, *T. longispicularis*, *T. probolurus*, *T. retortaefoemis*, *T. rugatus,* and *T. vitrinus*. According to the *in silico* analysis, the PCR product size was estimated to be approximately 100 bp. Optimal primer annealing temperature for empirical specificity of PCR was obtained by temperature-gradient PCR. Our findings indicated that 52 °C is optimal annealing temperature for the pair primer suitably able to amplify the specific region in the ITS-2 sequence of genomic DNA. No amplification was found in template controls. All the results showed that the PCR-HRM assay could be highly specific for targeting the *Trichostrongylus* spp.

Thirty DNA extractions were amplified using the pair primer in PCR reactions. No PCR product was found in gel electrophoresis for sample

Table 1
Trichostrongylus spp. detected via both PCR-sequencing and PCR-HRM assays (N = 30).

Tm	HRM group	Identity (%)	Coverage (%)	Subject (top score)	Sample (query)
76.82	G1	100	99	*T. colubriformis* (MH581147)	1
76.47	G1	100	98	*T. colubriformis* (MH581147)	2
76.48	G1	100	100	*T. colubriformis* (MH581147)	3
76.58	G1	100	100	*T. colubriformis* (MH581147)	4
76.14	G1	100	100	*T. colubriformis* (MH581147)	5
76	G1	98.08	100	*T. vitrinus* (KR020012)	6
76.18	G1	100	98	*T. colubriformis* (MH581147)	7
76.13	G1	100	98	*T. colubriformis* (MH581147)	8
76.31	G1	99.02	98	*T. colubriformis* (MH581147)	9
76.11	G1	99.02	98	*T. colubriformis* (MH581147)	10
76.63	G1	99.02	99	*T. colubriformis* (MH581147)	11
76.09	G1	100	98	*T. colubriformis* (MG770110)	12
76.5	G1	99.01	97	*T. colubriformis* (MH581147)	13
76.35	G1	100	100	*T. colubriformis* (MH581147)	14
76.52	G1	98.04	98	*T. colubriformis* (MH581147)	15
78.56	G3	99.02	98	*T. colubriformis* (MH581147)	16
76.64	G1	91.35	100	*T. colubriformis* (MH581147)	17
76.12	G1	100	100	*T. colubriformis* (MH581147)	18
76.41	G1	91.09	96	*T. colubriformis* (MH581147)	19
76.41	G1	98.04	98	*T. colubriformis* (MH581147)	20
75.62	G4	99	96	T. capricola (JF276023)	21
76.32	G2	96.12	100	*T. vitrinus* (MH047842)	22
76.5	G2	100	100	*T. vitrinus* (KC998732)	23
	Negative	—	—	—	24
76.36	G1	99.04	100	*T. colubriformis* (MH581147)	25
76.45	G1	99.04	100	*T. colubriformis* (MH581147)	26
76.39	G1	100	100	*T. colubriformis* (MH581147)	27
76.22	G2	99.04	100	*T. vitrinus* (KR020012)	28
76.31	G1	100	100	*T. colubriformis* (MH581147)	29
76.33	G1	100	100	*T. colubriformis* (MH581147)	30

24, indicating the inability of the primer pair to anneal to the ITS-2 sequence (Fig. 1B). Twenty-nine PCR products were purified for sequencing. Species identification was conducted by Sanger sequencing of the ITS-2 PCR products for all samples. Results from BLASTn search identified three *Trichostrongylus* spp. in the samples, including *T. colubriformis*, (n = 24), *T. vitrinus* (n = 4), and *T. capricola* (n = 1) (Table 1).

Afterwards, all *Trichostrongylus* DNA samples (n = 30) were subjected to PCR-HRM analysis. Normalization regions of 72.7–73.7 °C and 79.1–80.1 °C were used. Fig. 2 indicates the normalized HRM curves for the amplicons from the *Trichostrongylus* spp. A sharp decrease in fluorescence was detected in denatured DNA for each species, as shown in the normalized fluorescence curves (Fig. 2), which was consistent with its respective melting profile (Fig. 3). During PCR-HRM, genomic DNA molecule from each species relatively produced a unique melting plot which was useable to distinguish each species from other species. After PCR-HRM analysis, the samples were divided into four groups, including groups G1 (samples 1–16, 18–20, 25–27, 29, and 30), G2 (samples 22, 23, and 28), G3 (sample 17), and G4 (sample 21). Moreover, no PCR-HRM result was found for sample 24 (Table 1). The species-specific identification of *Trichostrongylus* isolates by the PCR-HRM assay was approximately consistent with sequencing analysis.

4. Discussion

Nowadays, diagnosis of *Trichostrongylus* infection is challenging in the control strategy due to difficult identification of various *Trichostrongylus* spp. from adult worms. Although diagnosis of *Trichostrongylus* infection is essential for the control of parasitic infections, current methods, such as morphological assays used for the detection of this parasite, are normally predicated on the observations of light microscopy. More importantly, such methods possess some disadvantages [39], such as being time-consuming, inability to provide quantitative data [40,41], and decreased possibility to distinguish the species belonging to a genus. Of note, most studies highlighted the significant role of molecular techniques as valuable tools to identify and discriminate *Trichostrongylus* spp. Some PCR-based approaches were developed for epidemiologic studies, based on amplification of ribosomal DNA ITS-1 or ITS-2 as an identification target [11,15,16,42–44] while there is no PCR-HRM method to distinguish *Trichostrongylus* spp. Therefore, the present study explored the use of PCR-HRM assays to identify species of *Trichostrongylus* adult worms.

Classification of *Trichostrongylus* spp. based on conventional morphological methods is relatively reliable for *Trichostrongylus* males. However, these methods are laborious and lack the ability to recognize female worms [15]. The conventional methods for diagnosis of different *Trichostrongylus* spp. are time-consuming, suffer from contamination risks, and remain difficult to distinguish species that are morphologically similar to each others [45,46].

In the present study, we successfully developed a specific real-time PCR and HRM technique on ITS-2 gene for rapid and precise differentiation of *Trichostrongylus* spp. using only a single primer pair. The primer set for *Trichostrongylus* was designed to amplify an internal region harboring the ITS-2, as a genetic marker for the detection and differentiation of *Trichostrongylus* [11,44].

The average T_m variation obtained by melting curve analysis was about 1 °C. This result showed a sufficient ability and reliability for distinguishing these four parasites. Following previous studies and because of the importance of *Trichostrongylus* infections worldwide, there is a need for more comprehensive studies on this parasite and the diagnosis of its species. The rDNA is among the most frequently-used targets for PCR, due to their high copy number and their ability to display nucleotide sequence variability among species, facilitating identification of parasite species [47].

In this investigation, we evaluated the accuracy of our molecular approach by direct sequencing of all amplicons from sheep samples. Our results showed that PCR-HRM could be a suitable and rapid tool for the diagnosis of Trichostrongyliasis with the ability to differentiate

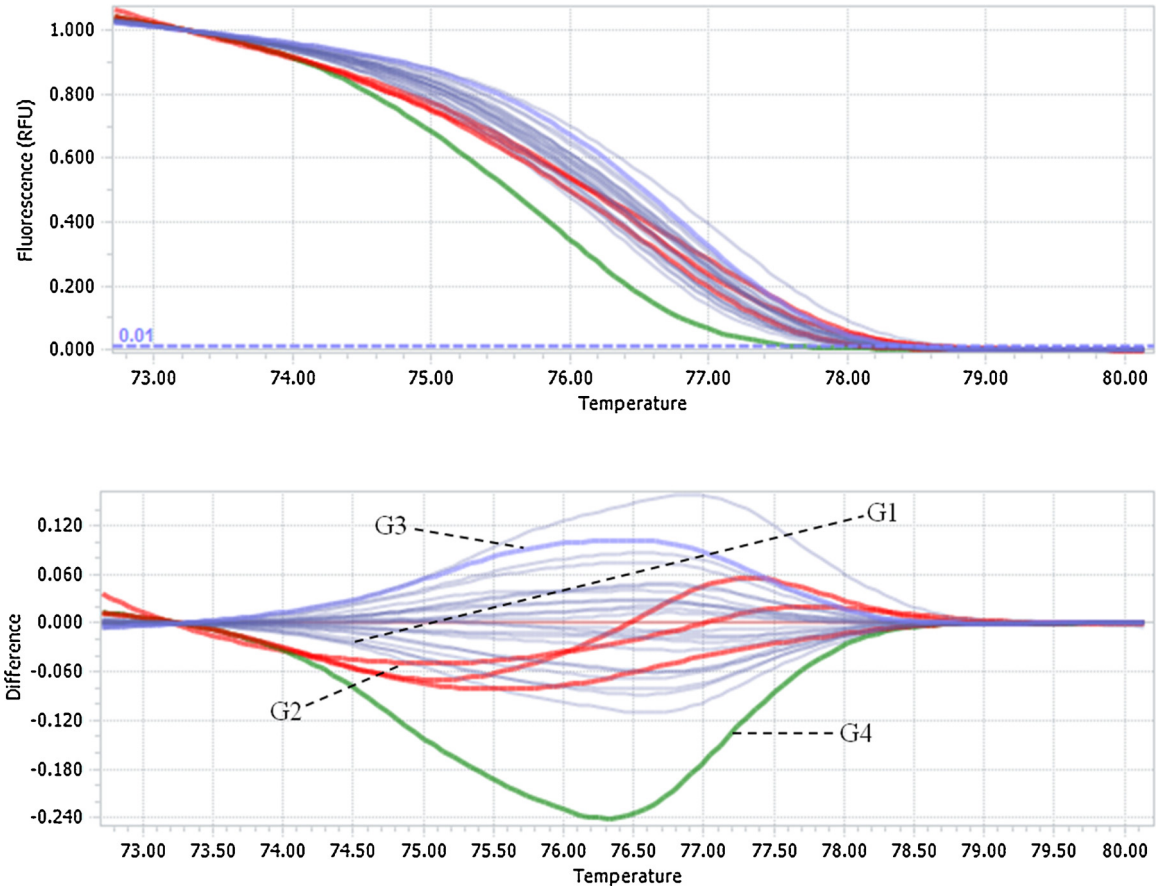

Fig. 2. Representative profiles of the melting curves (upper) and differences plots (lower) of ITS-2 amplicons for *T. capricola* (green, G4), *T. colubriformis* (gray and blue, G1 and G3) and *T. vitrinus* (red, G2). Normalized fluorescence is plotted against temperature (°C).

various species of *Trichostrongylus*.

Therefore, to assess and promote the diagnosis of various *Trichostrongylus* spp., the present study explored the use of PCR-HRM assays to detect adult parasites. The molecular protocol applied in this study was associated with amplification of ITS-2 regions of rDNA from different *Trichostrongylus* spp., and analysis of melting curves obtained from them. The PCR-HRM used for ITS-2 showed a fair potential for the detection of this parasite species. In recent years, PCR-based techniques have been certified for species identification and phylogenetic analysis of *Trichostrongylus* nematodes in ruminants worldwide, [16,48–51] and PCR-HRM can be helpful for these purposes. This assay needs to be evaluated on a large sample size in various geographical regions in the world especially in endemic areas. The advantage, however, is that the PCR-HRM technique was performed for the first time in the world for molecular identification of *Trichostrongylus* spp.

Since Trichostrongyliasis is one of the most prevalent zoonotic parasitic diseases worldwide, it is expected that a cost-effective and easy to set-up assay could help better detect *Trichostrongylus* spp. infections.

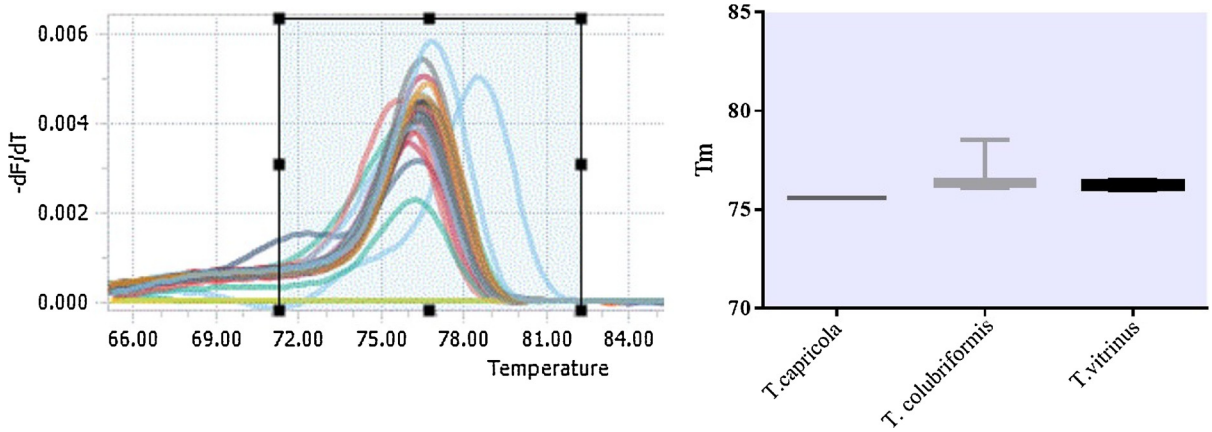

Fig. 3. Representative profiles of the melting peaks of ITS-2 amplicons for Trichostrongylus spp. (left). Box plot shows melting curve temperatures (T_m) for each Trichostrongylus spp. in PCR-HRM analysis (right).

5. Conclusion

The developed PCR-HRM technique in the present study permits to diagnose *Trichostrongylus* spp. among the four most relevant groups, including *T.colubriformis. T.capricula, T.vitrinus,* and *T.probblorous*. Consequently, this powerful, easy, low-cost, and sensitive assay has the ability to achieve suitable results less than two hours. The developed molecular procedure combined with microscopy would allow a more careful screening in animals for this disease.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

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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.molbiopara.2020.111260.

References

[1] F. Mavrot, H. Hertzberg, P. Torgerson, Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis, Parasit. Vectors 8 (1) (2015).
[2] G. Coulson, J.K. Cripps, S. Garnick, V. Bristow, I. Beveridge, Parasite insight: assessing fitness costs, infection risks and foraging benefits relating to gastrointestinal nematodes in wild mammalian herbivores, Philos. Trans. R. Soc. B: Biol. Sci. 373 (1751) (2018).
[3] F.V. Velde, J. Charlier, E. Claerebout, Farmer behavior and gastrointestinal nematodes in ruminant livestock-uptake of sustainable control approaches, Front. Vet. Sci. 5 (October) (2018).
[4] A. Al-Rofaai, W.A. Rahman, S.F. Sulaiman, Z.S. Yahaya, In vitro ovicidal and larvicidal activity of methanolic leaf extract of Manihot esculenta (cassava) on susceptible and resistant strains of Trichostrongylus colubriformis, Vet. Parasitol. 190 (1-2) (2012) 127–135.
[5] L. Roberts, J. Janovy, Foundations of Parasitology, 8 ed., McGraw-Hill Higher Education, Boston, USA, 2009.
[6] V.J. Adams, M.B. Markus, J.F. Adams, E. Jordaan, B. Curtis, M.A. Dhansay, C.C. Obihara, J.E. Fincham, Paradoxical helminthiasis and giardiasis in Cape Town, South Africa: epidemiology and control, Afr. Health Sci. 5 (2) (2005) 131–136.
[7] A.M. el-Shazly, H.A. el-Nahas, M. Soliman, D.M. Sultan, A.H. Abedl Tawab, T.A. Morsy, The reflection of control programs of parasitic diseases upon gastrointestinal helminthiasis in Dakahlia Governorate, Egypt, J. Egypt. Soc. Parasitol. 36 (2) (2006) 467–480.
[8] S. Lattes, H. Ferte, P. Delaunay, J. Depaquit, M. Vassallo, M. Vittier, S. Kokcha, E. Coulibaly, P. Marty, Trichostrongylus colubriformis nematode infections in humans, France, Emerging Infect. Dis. 17 (7) (2011) 1301–1302.
[9] E. Ghadirian, F. Arfaa, Present status of trichostrongyliasis in Iran, Am. J. Trop. Med. Hyg. 24 (6 Pt 1) (1975) 935–941.
[10] M. Tongson, S. Eduardo, CRC Handbook Series in Zoonoses: Section C Parasitic Zoonoses, CRC Press, USA, 1982.
[11] F. Perandin, E. Pomari, C. Bonizzi, M. Mistretta, F. Formenti, Z. Bisoffi, Assessment of real-time polymerase chain reaction for the detection of trichostrongylus spp. DNA from human fecal samples, Am. J. Trop. Med. Hyg. 98 (3) (2018) 768–771.
[12] V.M. Nantulya, Molecular diagnosis of parasites, Experientia 47 (2) (1991) 142–145.
[13] B. Singh, Molecular methods for diagnosis and epidemiological studies of parasitic infections, Int. J. Parasitol. 27 (10) (1997) 1135–1145.
[14] R.B. Gasser, Molecular technologies in parasitology, with an emphasis on genomic approaches for investigating parasitic nematodes, Parassitologia 48 (1-2) (2006) 9–11.
[15] M. Sharifdini, Z. Heidari, Z. Hesari, S. Vatandoost, E.B. Kia, Molecular phylogenetics of trichostrongylus species (Nematoda: Trichostrongylidae) from humans of Mazandaran Province, Iran, Korean J. Parasitol. 55 (3) (2017) 279–285.
[16] R. Ghasemikhah, M. Sharbatkhori, I. Mobedi, E. Kia, M.F. Harandi, H. Mirhendi, Sequence analysis of the second internal transcribed spacer (ITS2) region of rDNA for species identification of trichostrongylus nematodes isolated from domestic livestock in Iran, Iran. J. Parasitol. 7 (2) (2012) 40–46.
[17] D. Liu, Molecular Detection of Human Parasitic Pathogens, 1 ed., Taylor & Francis CRC Press, USA, 2012.
[18] M. Erali, K.V. Voelkerding, C.T. Wittwer, High resolution melting applications for clinical laboratory medicine, Exp. Mol. Pathol. 85 (1) (2008) 50–58.
[19] J.L. Montgomery, L.N. Sanford, C.T. Wittwer, High-resolution DNA melting analysis in clinical research and diagnostics, Expert Rev. Mol. Diagn. 10 (2) (2010) 219–240.
[20] C.T. Wittwer, High-resolution DNA melting analysis: advancements and limitations, Hum. Mutat. 30 (6) (2009) 857–859.
[21] A. Pangasa, A.R. Jex, B.E. Campbell, N.J. Bott, M. Whipp, G. Hogg, M.A. Stevens, R.B. Gasser, High resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans, Mol. Cell. Probes 23 (1) (2009) 10–15.
[22] M. Bienz, M. Siles-Lucas, N. Muller, Use of a novel DNA melting profile assay for the identification of PCR-amplified genomic sequences encoding for variant-specific surface proteins from the clonal GS/M-83-H7 line of Giardia lamblia, Parasitol. Res. 87 (12) (2001) 1011–1015.
[23] P. Zhang, Y. Liu, M. Alsarakibi, J. Li, T. Liu, Y. Li, G. Li, Application of HRM assays with EvaGreen dye for genotyping Giardia duodenalis zoonotic assemblages, Parasitol. Res. 111 (5) (2012) 2157–2163.
[24] D. Talmi-Frank, A. Nasereddin, L.F. Schnur, G. Schonian, S.O. Toz, C.L. Jaffe, G. Baneth, Detection and identification of old world Leishmania by high resolution melt analysis, PLoS Negl. Trop. Dis. 4 (1) (2010) e581.
[25] B.S. Robinson, P.T. Monis, P.J. Dobson, Rapid, sensitive, and discriminating identification of Naegleria spp. by real-time PCR and melting-curve analysis, Appl. Environ. Microbiol. 72 (9) (2006) 5857–5863.
[26] K. Kassaza, D.J. Operario, D. Nyehangane, K.C. Coffey, M. Namugosa, L. Turkheimer, P. Ojuka, P. Orikiriza, J. Mwanga-Amumpaire, F. Byarugaba, J. Bazira, J.L. Guler, C.C. Moore, Y. Boum 2nd, Detection of Plasmodium species by high-resolution melt analysis of DNA from blood smears acquired in Southwestern Uganda, J. Clin. Microbiol. 56 (1) (2018).
[27] E. Murillo, C. Muskus, L.A. Agudelo, I.D. Velez, F. Ruiz-Lopez, A new high-resolution melting analysis for the detection and identification of Plasmodium in human and Anopheles vectors of malaria, Sci. Rep. 9 (1) (2019) 1674.
[28] J. Li, G.H. Zhao, R. Lin, D. Blair, H. Sugiyama, X.Q. Zhu, Rapid detection and identification of four major Schistosoma species by high-resolution melt (HRM) analysis, Parasitol. Res. 114 (11) (2015) 4225–4232.
[29] H. Sady, H.M. Al-Mekhlafi, R. Ngui, W.M. Atroosh, A.K. Al-Delaimy, N.A. Nasr, S. Dawaki, A.M. Abdulsalam, I. Ithoi, Y.A. Lim, K.H. Chua, J. Surin, Detection of Schistosoma mansoni and Schistosoma haematobium by real-time PCR with high resolution melting analysis, Int. J. Mol. Sci. 16 (7) (2015) 16085–16103.
[30] V.H. Pena, G.J. Fernandez, A.M. Gomez-Palacio, A.M. Mejia-Jaramillo, O. Cantillo, O. Triana-Chavez, High-resolution melting (HRM) of the cytochrome B gene: a powerful approach to identify blood-meal sources in Chagas disease Vectors, PLoS Negl. Trop. Dis. 6 (2) (2012) e1530.
[31] R. Ngui, Y.A. Lim, K.H. Chua, Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis, PLoS One 7 (7) (2012) e41996.
[32] S. Areekit, P. Kanjanavas, A. Pakpitchareon, P. Khawsak, S. Khuchareontaworn, T. Sriyaphai, K. Chansiri, High resolution melting real-time PCR for rapid discrimination between Brugia malayi and Brugia pahangi, J. Med. Assoc. Thai. Chotmaihet thangphaet 92 (Suppl 3) (2009) S24–8.
[33] T. Thanchomnang, P.M. Intapan, C. Tantrawatpan, V. Lulitanond, S. Chungpivat, P. Taweethavonsawat, W. Kaewkong, O. Sanpool, P. Janwan, W. Choochote, W. Maleewong, Rapid detection and identification of Wuchereria bancrofti, Brugia malayi, B. pahangi, and Dirofilaria immitis in mosquito vectors and blood samples by high resolution melting real-time PCR, Korean J. Parasitol. 51 (6) (2013) 645–650.
[34] A. Hosseini-Safa, M.B. Rokni, S.H. Mosawi, P. Heydarian, H. Azizi, A. Davari, M. Aryaiepour, High-resolution melting analysis as an appropriate method to differentiate between Fasciola hepatica and F. gigantica, Iran. J. Public Health (2019).
[35] F. Eskandari, M.A. Mohaghegh, F. Mirzaei, M. Ghomashlooyan, S.H. Hejazi, Molecular characteristics of Echinococcus granulosus strains isolated from Iranian camel using high resolution melting analysis of atp6 and cox1 genes, Avicenna J. Clin. Microbiol. Infect. 5 (2) (2018) 14–19.
[36] A. Rojas, G. Segev, A. Markovics, I. Aroch, G. Baneth, Detection and quantification of Spirocerca lupi by HRM qPCR in fecal samples from dogs with spirocercosis, Parasit. Vectors 10 (1) (2017) 435.
[37] R. Ghasemikhah, H. Mirhendi, E.B. Kia, G. Mowlavi, H. Sarmadian, B. Meshgi, B. Golestan, I. Mobedi, Morphological and morphometrical description of Trichostrongylus species isolated from domestic ruminants in Khuzestan Province, Southwest Iran, Iran. J. Parasitol. 6 (3) (2011) 82–88.
[38] R.C. Anderson, Nematode Parasites of Vertebrates: Their Development and Transmission, 2nd ed., CABI Publishing, Wallingford UK, 2000.
[39] S.J.M. Preston, M. Sandeman, J. Gonzalez, D. Piedrafita, Current status for gastrointestinal nematode diagnosis in small ruminants: where are we and where are we going? J. Immunol. Res. 2014 (2014).
[40] M. Ndao, Diagnosis of parasitic diseases: old and new approaches, Interdiscip. Perspect. Infect. Dis. 2009 (2009) 278246.
[41] S. Momčilović, C. Cantacessi, V. Arsić-Arsenijević, D. Otranto, S. Tasić-Otašević, Rapid diagnosis of parasitic diseases: current scenario and future needs, Clin. Microbiol. Infect. 25 (3) (2019) 290–309.
[42] T.S. Yong, J.H. Lee, S. Sim, J. Lee, D.Y. Min, J.Y. Chai, K.S. Eom, W.M. Sohn, S.H. Lee, H.J. Rim, Differential diagnosis of Trichostrongylus and hookworm eggs via PCR using ITS-1 sequence, Korean J. Parasitol. 45 (1) (2007) 69–74.
[43] M. Sato, T. Yoonuan, S. Sanguankiat, S. Nuamtanong, T. Pongvongsa, I. Phimmayoi, V. Phanhanan, B. Boupha, K. Moji, J. Waikagul, Short report: human Trichostrongylus colubriformis infection in a rural village in Laos, Am. J. Trop. Med. Hyg. 84 (1) (2011) 52–54.
[44] I. Phosuk, P.M. Intapan, O. Sanpool, P. Janwan, T. Thanchomnang,

K. Sawanyawisuth, N. Morakote, W. Maleewong, Molecular evidence of Trichostrongylus colubriformis and Trichostrongylus axei infections in humans from Thailand and Lao PDR, Am. J. Trop. Med. Hyg. 89 (2) (2013) 376–379.
[45] C.F. Amar, C.L. East, J. Gray, M. Iturriza-Gomara, E.A. Maclure, J. McLauchlin, Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993-1996), Eur. J. Clin. Microbiol. Infect. Dis. 26 (5) (2007) 311–323.
[46] K.L. Muldrew, Molecular diagnostics of infectious diseases, Curr. Opin. Pediatr. 21 (1) (2009) 102–111.
[47] J.J. Verweij, C.R. Stensvold, Molecular testing for clinical diagnosis and epidemiological investigations of intestinal parasitic infections, Clin. Microbiol. Rev. 27 (2) (2014) 371–418.
[48] H. Hoste, N.B. Chilton, R.B. Gasser, I. Beveridge, Differences in the second internal transcribed spacer (ribosomal DNA) between five species of Trichostrongylus (Nematoda: Trichostrongylidae), Int. J. Parasitol. 25 (1) (1995) 75–80.
[49] D.N. Kuznetsov, N.A. Kuznetsova, Sequences of the second internal transcribed spacer of ribosomal DNA for three species of Trichostrongylus (Nematoda: Trichostrongylidae) from sheep in Russia, Helminthologia 44 (2) (2007) 43–46.
[50] R.B. Gasser, N.B. Chilton, H. Hoste, L.A. Stevenson, Species identification of trichostrongyle nematodes by PCR-linked RFLP, Int. J. Parasitol. 24 (2) (1994) 291–293.
[51] T.K. Tan, C. Panchadcharam, V.L. Low, S.C. Lee, R. Ngui, R.S. Sharma, Y.A. Lim, Co-infection of Haemonchus contortus and Trichostrongylus spp. among livestock in Malaysia as revealed by amplification and sequencing of the internal transcribed spacer II DNA region, BMC Vet. Res. 10 (2014) 38.