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# Accentuated Molecular Detection Technique to Segregate and Identify Helminths of Fish through High Resolution Melting (HRM) Analysis

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## Abstract

Real-time PCR coupled with high-resolution molecular (HRM) analysis was conducted to target the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA. The latter acted as a genetic marker to identify and distinguish two anisakid and one cucullanid species parasitizing marine and freshwater fish. Unique and distinct characteristics of HRM patterns were produced for each of the three roundworms investigated. The melt profiles and threshold of the cycles (Ct values), at which amplification commenced, for *Anisakis simplex* (Rudolphi), *Contrcaecum osculatum* (Rudolphi) Dujardin, *Contrcaecum* sp. and *Dacnitoides cotylophora* (Ward and Magath) were diagnostic for species. The molecular analyses by sequencing and comparing the internal transcribed spacer (ITS) of the ribosomal DNA of Dacnitoides, Anisakis and Contrcaecum assays established their distinct identity. The present investigation propagates molecular-phylogenetic and morpho-molecular analysis to characterize diagnostic constituents of cucullanid and anisakid roundworms.

**Keywords:** Molecular detection; Single stranded DNA; Helminths; Fish; RT-PCR; Cucullanid; Anisakid

## Introduction

The molecular diagnostic procedures based on RT-PCR have proved to be reliable biotechnological tool from the standpoint of segregation of species and other taxa. Their potential to detect multiple pathogens simultaneously in one setting has put these on a higher pedestal of clinical significance as well. The asymptomatic infections of invasive parasitic protozoans were detected in earlier investigations [1,2]. The inquisitiveness about such infections could thus be PCR-coupled technology has been scarcely used for detection and diagnosis of helminth infections, particularly of zoonotic significance.

The analysis conducted by real-time PCR with SYBR Green1 melting curve analysis in this study proved to be an effective and sensitive method for specific detection and identification of pathogenic parasites of zoonotic significance.

The conservation of specific products at the required specific temperature to generate fluorescence data, and simultaneous denaturation of non-specific products hides the disadvantageous binding characteristic of the dye with all the double stranded DNA comprising primer dimers and non-specific products. Therefore, this fluorescence characteristic and the associated melting point variants have been attributed immense taxonomic significance for taxa differentiation.

The nucleic acid sequence elongation and its conformation is sensitive to the melting point of amplicon. Thus, the melting point curve analysis has thus provided an opportunity to correlate it with sequence length variations to determine species-specific sequence variation. Ct values have been recognized to be the sensitive and specific tools that banished the requirement of electrophoresis of amplicons, and instead assisted the process to detect the adequate number of cycles required to cross the threshold at the time of initiation of the amplification cycle, so that ultimately relative or absolute quantification of nucleic acids could be attained.

The assistance of SEM applications, as well as RT-PCR technology for molecular assessment, the systematic and taxonomic segregation of the three genera viz., Dacnitoides, Anisakis and Contrcaecum has been facilitated. The innovative molecular applications have resulted into fast track diagnostics to resolve issues of biodiversity and Systematic Parasitology [3].

It was recently highlighted [4] that the nucleotide sequence length and barcode structure are a function of the melting point of an amplicon. Therefore, it is obvious that the diversity of sequence variation within and among samples can be determined by the melting point analysis, thereby confirming the use of melting curve analysis as a tool to establish distinct identity of individual amplicons comprising contrasting sequences [5].

Since marine fish are the main reservoirs of the nematodes of Anisakidae in the coastal ecosystem at Goa, whose larvae were quite often difficult to discern with from adult worms morphologically, after real-time PCR amplification, HRMA allowed to discriminate clearly the three species according to the different melting temperatures of the amplicons, although they have an identical number of bases [6]. Melting profiles showed minimal variability within samples from the same group. The specificity of the amplification was confirmed by sequence analysis, thus proving that the clustering pattern presented in the curves was representing two different species, as effectively as DNA sequencing. The cost effectiveness of real-time PCR–HRMA is comparable to, or cheaper than, conventional PCR and sequencing, with a very rapid postamplification detection step [7].

Although the recent investigations to develop advanced methods for

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detection and assessment of abundance of a variety of trematodes and other parasites, including flatworms, without collection of hosts [8-11], the application of PCR-based tests [12], standard field protocols need be established as yet, particularly to clearly understand the environmental persistence of DNA [13]. However, there are disadvantages too in the employment of eDNA methods, as they are also prone to false negatives if DNA degradation occurred due to the errors in sampling on error in calculation of timings of infection outbreak or due to spatial or temporal implications.

## Materials and Methods

A more rapid, close-tube, melt peak assays were generated from a variety of anisakid nematodes from marine and freshwater fish using RT-PCR technology. The adult roundworms of *Anisakis simplex* (Rudolphi) and *Contrcaecum osculatum* (Rudolphi) Dujardin were collected from *Sillago sihama*, and a sciaenid, *Johnius dussumieri*, respectively, during February through November, 2014. The larval *C. osculatum* were recovered from *S. sihama*, and of *Contrcaecum* from *J. dussumieri*. Simultaneously, freshwater catfish, Rita rita was examined during this period from Gangetic riverine ecosystem at Fatehpur, Uttar Pradesh to collect the specimens of Dacnitoides cotylophora (Ward and Magath). The rationale for selecting two anisakids and one cucullanid species for the study was the extension of range of infectivity by the roundworms of Anisakidae and related groups of worms, from marine habitat in the fish hosts of areas of Central West coast of India at Goa to the freshwater fish hosts of Gangetic riverine ecosystems [14].

Total genomic DNA was extracted from these worms, using DNeasy Tissue Kit (Qiagen) in a final buffer volume of 50 µm. A 950 base pair (bp) fragment of the Inter Transcribed Spacer – 2 gene from the extracted DNA was amplified using primers SS2, 5'-TTGCAGACACATTGAGCACT-3'; and NC2, 5'-TTAGTTTCTTTTCCTCCGCT-3' [15] using a Thermal Cycler IQ5 Real Time PCR Detection System Biorad Laboratories Inc., Hercules, CA, USA. The total PCR volume was 20 um and contained 1 microlitre template DNA, 10 micorlitre SYBR Green ER Supermix and 0.4 microlitre of each primer (0.2 micromolar). Amplification was performed using the cycling profile 94°C initial denaturation (5min), 35 cycles of 94°C denaturation (30 sec), 53°C annealing (30 sec) and 72°C extension (30 sec)(extension), followed by a final extension at 72°C for 5 min. Fluorescence was measured during the annealing step of each cycle.

The fluorescence of intercalating dyes utilized in conducting qPCR, is attained on their being bound to double-stranded DNA (dsDNA), while the presence of single stranded DNA (ssDNA) does not induce these to fluoresce. Once the completion of amplification cycles is achieved, the melt curve is derived following the programmed instructions. The amount of fluorescence is then measured by thermal cycler at a preset temperature (usually above the primer $T_m$) at the termination of the qPCR run. The denaturation of dsDNA commenced to transform into single-stranded DNA resulting into simultaneous dissociation of the dye, after incremental increase in temperature, while simultaneous measure of fluorescence continued. The commencement of melting of the dsDNA ensures melting of unstable (i.e. G/C poor) prior to G/C rich regions until the temperature is sufficiently high to cause it to melt.

## Results

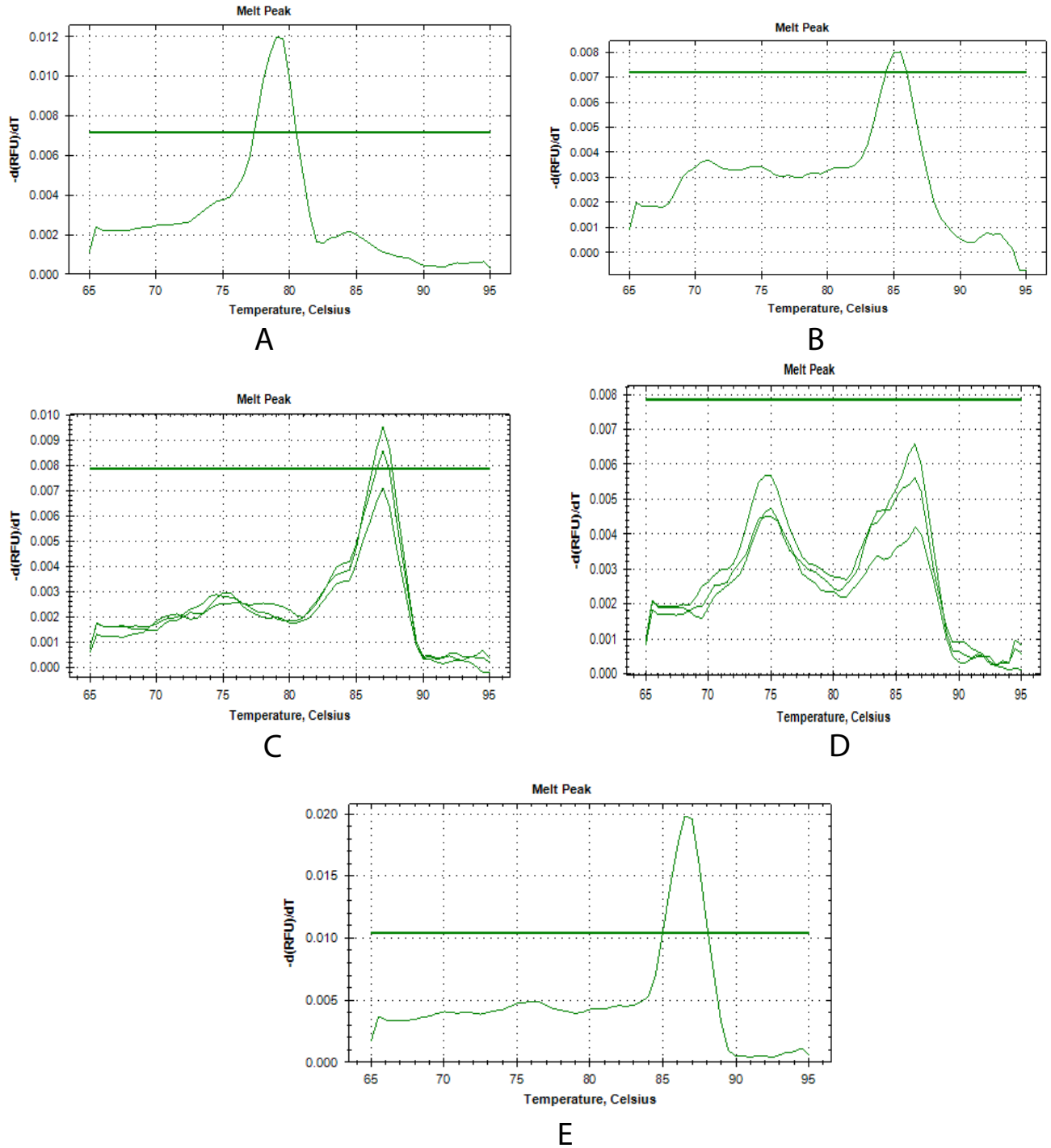
Threshold cycles (Ct values) for *A. simplex*, *C. osculatum* and *D. cotylophora* assays were consistent over time, and gave significantly comparable values that could enable to establish taxa differentiation. The findings of experimentation to denote peaks of melting point curves of ITS2 gene to distinctly establish the separate identity of adult specimens of *A. simplex* (Figure 1a, 79.59°C), adult (Figure 1b, 85.5°C) and larvae (Figure 1c, 87°C) of *Contracaecum* sp. larvae from *J. dussumieri* (Figure 1d, 86°C and 75°C), and adult worms of *D. cotylophora* (Figure 1e, 86.5°C) recovered from freshwater (R. rita) fish are shown in Figure 1e. As many as three replicates of each species were done for HRM analysis. But only the representative depiction of derivative melt curves all of these three replicates was included (Figure 1c and Figure 1d) to avoid repetition of illustrations, particularly because, these figures were for the larvae of the only genus *Contracaecum* that were collected from two different marine fish species, namely, larval *C. osculatum* from *S. sihama* (Figure 1c), as well as larval *C. osculatum* from *Johnius dussumieri* (Figure 1d). Simultaneously, the three replicates of aligned melt curves of larval *C. osculatum* from *S. sihama*, as well as larval *C. osculatum* from *Johnius dussumieri* have been presented in Figure 2c and Figure 2d, respectively.

Ct values analysed for *D. cotylophora* were 15.3-16.85°C, whereas these were 23.13-23.44°C for adult worms of *C. osculatum*, and 20.71-20.9°C for their larvae, and 11.72-14.02°C for the larvae of *Contracaecum* sp. These values were 18.9-19.19°C for adult specimens of *A. simplex*.

## Discussion

The application of RT-PCR technique not only enables detection and quantification in diagnostics, but also the identification of specific DNA sequences [16,17]. The use of real-time PCR for molecular diagnostics is attractive because it is objective, rapid, versatile and cost-effective and can be performed on small tissue samples. The melt peak and Tm values analysis enables identification of species. The finding on twin peaks at 75°C and 86.5°C in the present study were closer to the similar pattern of twin peaks of the specific melting temperature peaks at 80.1°C and 85°C recorded earlier [18], where a multiplex real time PCR assay was developed for the distinctive detection of *E. histolytica*, *Giardia lamblia* and *C. parvum* in stool samples. Two melting domains were reported recently [19] in a 550 bp amplicon of the hydroxytryptamine receptor 2A gene. Amplicons with multiple melting domains have been used to differentiate species of Giardia (660 bp amplicon from the *gdh* gene) [20] and Naegleria (350-400 bp amplicons from the intergenic spacer).

The intercalating dyes produce fluorescence only when bounded with double stranded DNA and the thermal cycler produces the melt curve after the process of amplification is completed. When the temperature is raised, the DNA denatures and thus the fluorescence decreases. The change in slope of the curve verses temperature obtains the melting curve. All the samples taken under study were identified using RT-PCR. The consistent Tms were obtained for replicates of each species, and each species has a different Tm value leading to its identification. The representative three replicates of melt point curves i.e. derivative curves (Figure 1c, *C. osculatum* larvae from *S. sihama*, Figure 1d, larval *C. osculatum* from *J. dussumieri*) as well as aligned curves (Figure 2c, *C. osculatum* larvae from *S. sihama*, Figure 2d, larval *C. osculatum* from *J. dussumieri*) have been shown, so as to avoid repetition of replicates each time, for all the three worms. This is particularly because the larvae of Contracaecum were encountered repeatedly in two different fish species in the marine ecosystem. The melting point is a function of GC content, therefore, the higher the GC content, the more would be the melting point and this forms the basis of differentiation between the different species. The results in this study showed highest melting point values for the adults (23.13-23.44°C) and larvae of *C. complanatum* (20.71-20.9°C), reflecting higher G:C content in these parasites of marine origin. However, the anisakid, *A. simplex*




**Figures 1.** Profiles of the melt peak curves (derivative melt curves) showing denaturation profile of ITS2 amplicons **a**: Adult Anisakis simplex from *Sillago sihama*, **b**: Adult *Contracaecum osculatum* from *Sillago sihama*, **c**: Larval *C. osculatum* from *Sillago sihama*, **d**: Larval *C. osculatum* from *Johnius dussumieri* **e**: Adult worms of *Dacnitoides cotylophora* recovered from freshwater Rita rita

(18.9-19.19°C), and the cucullanid species, *D. cotylophora* (15.3-16.85°C) had lower Tm values, while another *Contracaecum* sp. larvae had the lowest (11.72-14.02°C). The melting characteristics of ITS2 amplicons from all species were assessed by plotting two different curves (Figures 2a-2e). In the present study, the normalized fluorescence curves i.e. aligned melt curve (Figures 2a-2e) and derivative melt curve (Figure 2a) produced uniquely different plots that were easily distinguishable for each species. It would mean that although the melting profiles of different species (Tm) were very close to each other, they could clearly be discerned by the plotting of normalized melting curves (Figures 2e). The melting of DNA being a multi-state process (dsDNA and ssDNA) has been discussed already [21], and the possibility of an intermediate

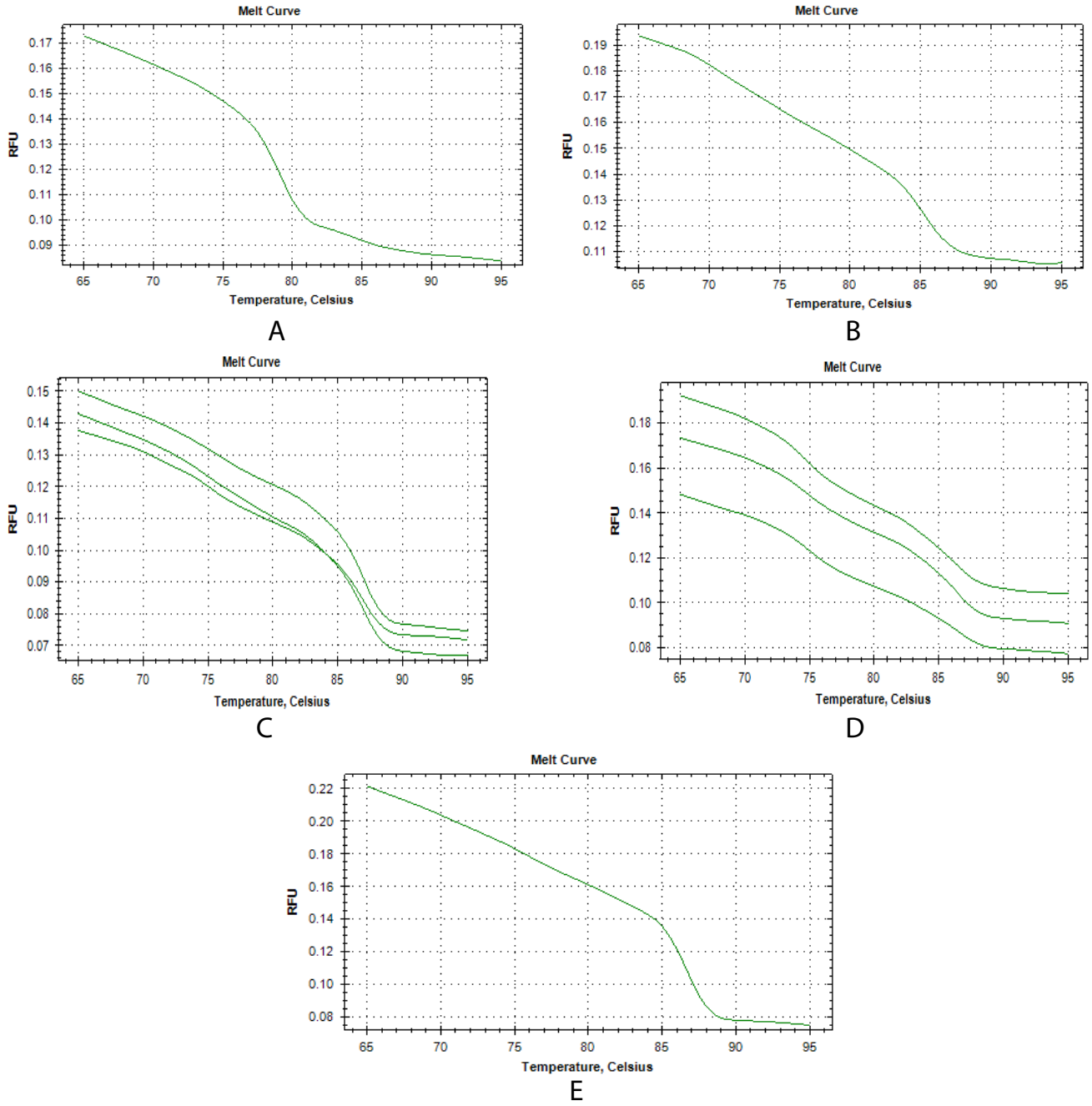



**Figure 2:** Profiles of the melt peak curves (aligned melt curves) showing denaturation profile of ITS2 amplicons. **a**: Adult Anisakis simplex from *Sillago sihama*, **b**: Adult *Contracaecum osculatum* from *Sillago sihama*, **c**: Larval *C. osculatum* from *Sillago sihama*, **d**: Larval *C. osculatum* from *Johnius dussumieri*, **e**: Adult worms of *Dacnitoides cotylophora* recovered from freshwater Rita rita.

state occurring to explain twin peaks as the melting events of the two amplicons, was put forth [21].

It has been recently asserted [22] that for species having almost similar melting curves and temperature-shifted fluorescence difference, a sharp decrease in fluorescence was detected in denatured DNA that was consistent for such species, with its respective melting point. The binding of intercalating dyes to any double stranded DNA is its drawback and henceforth melting point curve is a point for analysis, as non specific DNA is denatured at this point and specific DNA products remain intact. This associates the melting point to the composition and sequence of the nucleotides and characterizes sequence variation within the samples. The specific diagnosis made by the melting point curve is of importance in elucidating the genetic diversity, and will assist in study of differences in the biology, ecology and in the transmission of the parasites [4].

## Conclusion

Conclusively, therefore, the findings of this investigation revealed the potential role of real-time PCR in rapid diagnosis of cucullanids and agents of anisakidosis, as a step better than simple PCR technique.

It exhibited sensitivity compatible to that of traditional PCR but had the advantage of real-time detection of PCR products and the requirement of restriction digestion or sequence analysis for species differentiation or genotyping was kept in abeyance. Hence, real-time PCR has emerged as an ideal tool for screening for the detection and genotyping of parasites of zoonotic significance, in clinical laboratories.

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### References

1. Knobloch J, Mannweile E (1983) Development And Persistence Of Antibodies To Entamoeba Histolytica In Patients With Amebic Liver Abscess. Analysis of 216 cases. Ann J Trop Med Hyg 32: 727-732.
2. Wynants H, Ende VJ, Randria J, Gompel VA, Enden VE, et al. (1995) Diagnosis Of Amoebic Infection Of The Liver: Report Of 36 Cases. Ann Soc Belg Med Trop 75: 297-303.
3. Mattiucci S, Nascetti G (2008) Advances And Trends In The Molecular Systematic Of Anisakid Nematodes, With Implications For Their Evolutionary Ecology And Host-Parasite Co-Evolutionary Processes. Adv Parasitol 66: 47-148.
4. Gasser RB, Bott NJ, Chilton NB, Hunt P, Beveridge I (2008) Toward Practical, Dna-Based Diagnostic Methods For Parasitic Nematodes Of Livestock-Bionomic And Biotechnological Implications. Biotechnol Adv 26: 325-334.
5. Gasser RB (2006) Molecular Tools-Advances, Opportunities And Prospects. Vet Parasitol 136: 69-89.
6. Huver JR, Koprivnikar J, Johnson PTJ, Whyard S (2015) Development And Application Of An Edna Method To Detect And Quantify A Pathogenic Parasite In Aquatic Ecosystems. Ecol Appl 25: 991-1002.
7. Wongkamchai S, Mayoon B, Wanachiwanawin D, Foongladda S, Boitano JJ, et al. (2013) Real-Time Pcr With High-Resolution Melting Analysis For Diagnosis Of Lymphatic Filariasis. Proc J Int Trop Med Meet 3: 23-30.
8. Johnson M, Abs EL-Osta YG, Hu M, Gasser RB (1999) An Electrophoretic Tool For The Genetic Characterisation And Delineation Of Lungworms. Mol Cell Probes 18: 197–203.
9. Johnson PTJ, Kellermanns E, Bowerman J (2011) Critical Windows Of Disease Risk: Amphibian Pathology Driven By Developmental Changes In Host Resistance And Tolerance. Func Ecol 25: 726–734.
10. Goodman BA, Johnson PTJ (2011) Disease And The Extended Phenotype: Parasites Control Host Performance And Survival Through Induced Changes In Body Plan. PLoS One 6: e20193.
11. Goodman BA, Johnson PTJ (2011b) Ecomorphology And Disease: Understanding The Cryptic Effects Of Parasitism On Host Habitat Use, Thermoregulation, And Predator Avoidance. Ecology 92: 542-548.
12. Reinitz DM, Yoshino TP, Cole RA (2007) A Ribeiroia spp. (Class: Trematoda) -Specific PCR-based Diagnostic. J Parasitol 93: 1234-1238.
13. Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, et al. (2014) Environmental Conditions Influence Edna Persistence In Aquatic Systems. Environ Sci Technol 48: 1819-1827.
14. Malhotra SK (2014) Advances in Fish Parasitology – Emerging Paradigms of Parasitic Bioinvasion. Proc Train Wrkshp, Central Institute of Fisheries Education, Mumbai: 65-78.
15. Shamsi S, Suthar J (2016) Occurrence of Terranova Larval Types (Nematoda: Anisakidae) In Australian Marine Fish With Comments On Their Specific Idnetities. Peer J 4: e1722.
16. VanGuilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. BioTech 44: 619-626.
17. Winder LM, Phillips CB, Richards N, Corona FO, Hardwick S, et al. (2011) Evaluation of DNA Melting Analysis As A Tool For Species Identification. Method Ecol Evol 2: 312-320.
18. Verweij JJ, Blange RA, Templeton K, Schinkel J, Brienen EA, et al. (2004) Simultaneous Detection Of Entamoeba Histolytica, Giardia Lamblia, And Cryptosporidium Parvum In Fecal Samples Using Multiplex Real-Time PCR. J Clin Microbiol 42: 1220-1223.
19. Ngui R, Lim YAL, Chua KH (2012) Rapid Detection And Identification Of Human Hookworm Infections Through High Resolution Melting (Hrm) Analysis. PLoS One 7: e41996.
20. Monis PT, Giglio S, Saint CP (2005) Comparison Of Syto9 And Sybr Green I For Real-Time Polymerase Chain Reaction And Investigation Of The Effect Of Dye Concentration On Amplification And Dna Melting Curve Analysis. Anal Bio Chem 340: 24-34.
21. Fehlberg HF, Maciel BM, Albuquerque GR (2017) Identification and Discrimination of Toxoplasma Gondii, Sarcocystis spp., Neospora spp., and Cryptosporidium spp. by Righ-resolution Melting Analysis. PLoS ONE 12: e0174168.
22. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003) High-Resolution Genotyping By Amplicon Melting Analysis Using LC Green. Clin Chem 49: 853-860.

**Citation:** Jaiswal N, Tripathi R, Malhotra SK (2017) Accentuated Molecular Detection Technique to Segregate and Identify Helminths of Fish through High Resolution Melting (HRM) Analysis. Single Cell Biol 6: 163. doi:10.4172/2168-9431.1000163