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Ribo HRM — Detection of inter- and intra-species polymorphisms within ribosomal DNA by high resolution melting analysis supported by application of artificial allelic standards

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ABSTRACT

Ribo HRM, a single-tube PCR and high resolution melting (HRM) assay for detection of polymorphisms in the large subunit ribosomal DNA expansion segment V, was developed on a *Trichinella* model. Four *Trichinella* species: *T. spiralis* (isolates ISS3 and ISS160), *T. nativa* (isolates ISS10 and ISS70), *T. britovi* (isolates ISS2 and ISS392) and *T. pseudospiralis* (isolates ISS13 and ISS1348) were genotyped. Cloned allelic variants of the expansion segment V were used as standards to prepare reference HRM curves characteristic for single sequences and mixtures of several cloned sequences imitating allelic composition detected in *Trichinella* isolates.

Using the primer pair Tsr1 and Trich1bi, it was possible to amplify a fragment of the ESV and detect PCR products obtained from the genomic DNA of pools of larvae belonging to the four investigated species: *T. pseudospiralis*, *T. spiralis*, *T. britovi* and *T. nativa*, in a single tube Real-Time PCR reaction. Differences in the shape of the HRM curves of *Trichinella* isolates suggested the presence of differences between examined isolates of *T. nativa*, *T. britovi* and *T. pseudospiralis* species. No differences were observed between *T. spiralis* isolates. The presence of polymorphisms within the amplified ESV sequence fragment of *T. nativa T. britovi* and *T. pseudospiralis* was confirmed by sequencing of the cloned PCR products. Novel sequences were discovered and deposited in GenBank (GenBank IDs: JN971020–JN971027, JN120902.1, JN120903.1, JN120904.1, JN120906.1, JN120905.1).

Screening the ESV region of *Trichinella* for polymorphism is possible using the genotyping assay Ribo HRM at the current state of its development. The Ribo HRM assay could be useful in phylogenetic studies of the *Trichinella* genus.

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1. Introduction

Parasites belonging to the genus *Trichinella* can infect humans and animals causing trichinellosis. The infection can be fatal to humans and cause losses in animal production. The family Trichinellidae contains a single genus *Trichinella* divided into 8 species (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murelli*, *T. nelsonii*, *T. zimbabwensis*, *T. papuae*) and four genotypes whose taxonomic rank has not been resolved — T5, T6, T8 and T12 (Pozio et al., 2009). The phylogeny of the *Trichinella* genus is, to large extent, based on ribosomal DNA (rDNA) sequences, i.e. the ribosomal small-subunit DNA (SSU-rDNA) and internal transcribed spacer 2 (ITS-2) sequences (Zarlenga et al., 2006). Another region used for genotyping and phylogenetic analysis is the large subunit rDNA (lsrDNA) expansion segment V (ESV) (Zarlenga and Dame, 1992; La Rosa et al., 2001). The expansion segments are a part of 26S/28S lsrDNA and exist in all Eukaryotes. The ESV regions exhibit low levels of similarity between distantly related eukaryotic species and significant similarity at the species level (Hancock and Dover, 1988) and therefore could be a good model for designing assays allowing species or strain (isolate) identification. Because ESV sequences were not available for all *Trichinella* genotypes the fragment was of limited applicability in the phylogenetic analysis of *Trichinella* spp. so far (Krivokapich et al., 2008).

The aim of this study was development of a PCR HRM based method for the detection of polymorphisms, within the large subunit rDNA expansion segment V on the *Trichinella* model using DNA isolated from populations of parasites.

2. Materials and methods

2.1. Trichinella isolates

Muscle larvae belonging to four *Trichinella* species (*T. spiralis*, isolate codes ISS3 and ISS160; *T. nativa*, isolate codes ISS10 and ISS70; *T. britovi*, isolate codes ISS2 and ISS392; and *T. pseudospiralis*, isolate codes ISS13 and ISS1348), preserved in ethanol, were provided by the International Trichinella Reference Center, Rome, Italy. Isolate information is available on the web site www.iss.it/site/Trichinella/scripts/sear.asp.

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2.2. DNA extraction

The DNA was extracted from pools of larva (several hundred larvae) with the QIAamp DNA Mini kit® (Qiagen) according to the manufacturer's instructions for tissue extraction.

2.3. PCR

The PCR test was designed to be universal for the *Trichinella* genus and to amplify approximately 100–250 bp of the ESV (depending on the investigated *Trichinella* species), using the primer pair Tsr1/Trich1bis. The Tsr1 primer (CGAAAACATACGACAACTGC) was described by Zarlenga et al. (1999) and the Trich1bis primer (CTAAGAAAA CGGCGAAAGC) was designed for the present work and matched the genomic sequences of at least five *Trichinella* species as determined by BLAST analysis: *T. zimbabwensis* (FJ572074.1), *T. papuae* (FJ493495.1), *T. pseudospiralis* (S82661.1, S82660.1; S82658.1, S82657.1) *T. spiralis* (AF342803.1) and *Trichinella* T12 (EF623892.1).

The amplification was carried out in a Corbett 6000 thermal cycler. The PCR mix consisted of 12.5 μl 2× SensiMix™ HRM mix from Quantace, 2.5 μl of 2.5 μM primers (final concentration of each primer was 250 nM), 1 μl of EvaGreen™ stock solution provided with the SensiMix™ HRM kit and 1 μl of the pUC18 plasmid with cloned ESV fragment (0.01 ng) or *Trichinella* genomic DNA (approximately 100 ng), water was added to reach a final volume of 25 μl. PCR started with a polymerase activation phase at 95 °C for 10 min, then 35 cycles at 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 45 s. HRM was carried out immediately after the PCR at the temperature increment steps of 0.1 °C from 70 °C to 85 °C, for 3 s at each step. The results were analyzed with Rotor Gene 6000 software, version 1.7 build 85. The genotype identification confidence cut off value of 94% was set up in the genotyping module of the software.

2.4. PCR product cloning and sequencing

After the HRM analysis, the PCR products of *Trichinella* spp. amplification with primer pair Tsr1 and Trich1 bis were purified using Qiagen QIAquick PCR purification kit, phosphorylated with T4 polynucleotide kinase from Fermentas, subsequently the enzyme was heat inactivated, the reactions were carried out according to the manufacturer's recommendations. Phosphorylated PCR products were ligated to the blunt ended linear plasmid; digested with SmaI endonuclease, dephosphorylated with the shrimp alkaline phosphatase, pUC18 vector was used and the ligation reaction was carried out with Rapid DNA Ligation Kit (Fermentas) following the recommendations from the user's manual. Ligation products were transformed into *E. coli* strain DH5α, and the bacteria were plated on LB/ampicillin plates. Positive clones were identified by colony PCR with M13 forward and reverse primers. Overnight bacterial cell cultures from positive clones were grown on liquid LB medium supplemented with ampicillin. Plasmids were isolated using the Kucharczyk Plasmid Mini Kit and subjected to Sanger sequencing with M13 forward and reverse primers. Each of the sequences deposited in GenBank was determined by sequencing both strands of at least two clones obtained in the course of independent PCR and cloning experiments.

2.5. Artificial isolate standards

ESV region sequence variants (alleles) detected in every isolate and cloned into pUC18, were used as allelic standards for preparation of the reference HRM curves specific for single ESV sequence variants or mixtures of sequence variants mimicking the ESV region allelic composition (artificial allelic standards) characteristic for the investigated populations of *Trichinella* isolates and were described as Artificial Isolate Standards (AIS). The following AIS were prepared using single sequences cloned into pUC18: ISS10 AIS — ISS10-ESV-V1 (GenBank ID: JN971020) and ISS160 AIS (GenBank ID: AF342803.1; bases 2827–2845). For isolates in which multiple ESV sequence variants were detected the AIS were prepared by mixing equimolar amounts of pUC18 clones containing each of the sequence variants detected in an isolate; ISS2 AIS: ISS2-ESV-v1 (GenBank ID: JN971024) and ISS2-ESV-v1a (GenBank ID: JN971023), ISS392 AIS: ISS-392ESV-v1 (GenBank ID: JN971027), ISS-392ESV-v2 (GenBank ID: JN971026), ISS-392ESV-v1a (GenBank ID: JN971025), ISS70 AIS: ISS70-ESV-v1 (GenBank ID: JN971021) and ISS70-ESV-v2 (GenBank ID: JN971022).

2.6. Data analysis

Multiple sequence alignments were performed with ClustalX2 (Larkin et al., 2007) and MAFFT algorithm using Jalview software (Waterhouse et al., 2009) and subsequently were manually edited using SeaView (Gouy et al., 2010) and Jalview software. Genotype identification was performed using Rotor Gene 6000 software — version 1.7 build 85. For establishing the confidence of identification four replicates of HRM for each isolate (genomic or AIS) were used. The lowest confidence of identification obtained for one of the replicates was defined as the confidence of identification of an isolate. On the graphs the HRM curves as presented in "replicate view" — isolate and allele HRM curves are consensus curves calculated by Rotor Gene 6000 software from replicates of the performed HRM reaction.

3. Results

Using the primer pair Tsr1 and Trich1bi, it was possible to amplify fragments of the ESV and detect PCR products obtained from the genomic DNA of pools of larvae belonging to the four investigated species: *T. pseudospiralis*, *T. spiralis*, *T. britovi* and *T. nativa*, in a single tube Real-Time PCR reaction (Fig. 1). Differences in the shape of the HRM curves of *Trichinella* isolates suggested differences between isolates of the same species: *T. nativa* isolates ISS10 and ISS70 (Figs. 1 and 2), *T. britovi* isolates ISS2 and ISS392 (Figs. 1 and 3), *T. pseudospiralis* isolates ISS13 and ISS1348 (Fig. 1). Inter-isolate HRM curve shape differences were: pronounced for *T. nativa* isolates ISS10 and ISS70 (Figs. 1 and 2), moderate for *T. pseudospiralis* isolates ISS13 and ISS1348 (Fig. 1) and subtle for *T. britovi* isolates ISS2 and ISS392 (Figs. 1 and 3). No differences in HRM curves were observed for *T. spiralis* isolates (Fig. 1). In all species in which inter-isolate differences in HRM curves were visible i.e. *T. nativa* (Figs. 1 and 2), *T. britovi* (Figs. 1 and 3) and *T. pseudospiralis* (Fig. 1), the presence of polymorphisms within the amplified ESV sequence fragment was confirmed by sequencing cloned PCR products. The following, novel sequences were discovered: *T. nativa* isolate ISS10 — single sequence ISS10-ESV-V1 (GenBank JN971020), *T. nativa* isolate ISS70 — two sequences — ISS70-ESV-v1 (GenBank ID: JN971021) and ISS70-ESV-v2 (GenBank ID: JN971022), *T. britovi* isolate ISS2 — two sequences ISS2-ESV-v1 (JN971024) and ISS2-ESV-v1a (JN971023), *T. britovi* isolate ISS392 — three sequences — ISS392-ESV-v1 (JN971027), ISS392-ESV-v1a (JN971025), ISS392-ESV-v2 (JN971026), *T. pseudospiralis* ISS13 — three sequences ISS13-ESV-v1a, ISS13-ESV-v1, ISS13-ESV-v2 (JN120902.1, JN120903.1, JN120904.1), *T. pseudospiralis* isolate ISS1348 — two sequences ISS1348-ESV-v1 and ISS13-ESV-v2 (JN120906.1, JN120905.1). The sequences found in *T. pseudospiralis* ISS13 were different from the ones described previously (Zarlenga et al., 1996; La Rosa et al., 2001) in this isolate (Fig. 4). The alignment of the ESV sequences of the investigated *T. britovi* (isolates ISS2 and ISS392) and *T. nativa* (isolates ISS10 and ISS70) with a fragment of *Trichinella* T12 isolate ISS1826 ESV sequence (GenBank ID: EF623892.1) revealed the presence of a novel microsatellite region in *T. britovi*, *T. nativa* and *Trichinella* T12 (Fig. 5). The microsatellite composed of two stretches of TG repeats separated by a short, AT rich region was present in all analyzed sequences (Fig. 5). The AT rich region was identical within single *Trichinella* species and differed between the *T. britovi*, *T. nativa* and *Trichinella* T12 (Fig. 5).

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