Molecular identification and characterization of *Trichinella spiralis* from a leopard in India

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**ABSTRACT**

Present study describes species level identification of *Trichinella spiralis* of leopard origin from India using multiplex PCR and molecular characterization of the parasite based on sequencing of multiple genetic markers viz. 5S ribosomal DNA intergenic spacer region (5S ISR), partial mitochondrial large ribosomal subunit (Mt-lsr) and partial mitochondrial cytochrome c oxidase I (COI) genes. A single amplicons of 173 bp, indicative of *T. spiralis* was obtained in multiplex PCR. Further, specific PCR amplifications viz. 750 bp (5S ISR), 445 bp (Mt-lsr) and 850 bp (COI) were obtained for selected genetic markers. Homology search analysis of 5S-ISR, Mt-lsr gene and COI gene showed highest 99.6% identity with sequences originating from China (KT894074, *T. spiralis*), 98.6% similarity with *T. spiralis* China isolates (GU339127, GU339147) and 99.8% sequence homology with *T. spiralis* sequences originating from Belarus (MH119334), respectively. In the phylogenetic analysis, sequences of each selected genetic marker clustered together with published *T. spiralis* isolates only, which further confirmed species level identification of detected larvae as *T. spiralis*, although very few differences were noted with reference to relative positions. This is the first study from India, which provide molecular evidence on circulation of *T. spiralis* in wild animals.

**Key words:** Leopard, Multiplex PCR, Phylogeny, Species identification, *Trichinella spiralis*.

**INTRODUCTION**

Trichinellosis is one of the most widespread food-borne helminthic zoonosis, caused by a group of unusual nematodes belonging to the genus *Trichinella* (Pozio, 2007). It has been detected in domestic and wild animals of almost all continents excepting Antarctica (Pozio and Murrell, 2006). In domestic and wild animals, *Trichinella* sp. infection has been reported infrequently from India (Ram et al., 2018). The first outbreak of trichinellosis in human in India was reported from Uttarakhand (Sethi et al., 2010). Presently, there are 9 valid species and 3 genotypes (Korhonen et al., 2016) and these species and genotypes are morphologically indistinguishable (Karadjian et al., 2017) excepting for the presence or absence of nurse cell capsule (encapsulated or non-encapsulated clade). Correct identification of species is challenging and can be achieved by using molecular techniques (Pozio et al., 2009). Such methods include species-specific size difference of amplicons in multiplex PCR (Zarlenga et al., 1999) or by PCR based amplification and sequencing of different molecular markers (Wang et al., 2012; Franssen et al., 2015). Present study describes for the first time, the species level identification of *T. spiralis* of leopard origin from India using multiplex PCR assay and DNA sequencing of multiple genetic markers.

**MATERIALS AND METHODS**

Collection and isolation of *Trichinella* larvae from muscle specimens: The masseter, diaphragm, tongue and intercostals muscles of a leopard that died in the forest of District-Dehradun (Uttarakhand, India) were submitted to the Centre for Wildlife of ICAR-IVRI, Izatnagar for parasitological examination. These muscles were then examined at the Division of Parasitology for the detection of *Trichinella* spp. infection. Muscles were examined microscopically by muscle-press technique and artificial muscle digestion technique (acid-pepsin digestion using magnetic stirrer) as suggested by International Commission on Trichinellosis (ICT; http://www.trichinellosis.org) to record the presence and collection of *Trichinella* larvae. Finely chopped muscle (20g) of each type was digested and average *Trichinella*...
larval burden (larvae per gram of muscle, lpg) was calculated in the sediment following repeated washing with tap water.

**DNA extraction from larvae:** Genomic DNA was extracted from 3 batches of five individual *Trichinella* spp. larva as well as a pool of five larvae each, using commercial kit (Qiagen, Germany). Concentration of DNA was measured using spectrophotometer (Nanodrop) and same was stored at -20°C till use.

**Species level identification of Trichinella larvae:** Multiplex PCR assay, as recommended by ICT and European Union Reference Laboratory (Zarlenga et al., 1999), was used to identify *Trichinella* larvae at species level. Primers were custom synthesized and subjected to multiplex PCR assay using genomic DNA of individual and pooled larvae and multiplex PCR kit (Qiagen, Germany). All reactions were carried out in 25 μl volume. PCR amplified products were visualized following agarose gel electrophoresis (2%), under UV illumination in a Gel Documentation system (SYNGENE, UK).

**Molecular characterization of Trichinella sp.:** The DNA of individual larva was subjected to molecular characterization study targeting different established markers viz. 5S ribosomal DNA intergenic spacer region (5S rDNA-ISR), partial mitochondrial large ribosomal subunit (Mt-lsr) gene and partial mitochondrial cytochrome c oxidase 1 (COI) gene. Details of primer sequences are given in Table 2.

PCR amplification was carried out in 25μl volume comprising of 10X Dream Taq Green buffer (Thermo Scientific, USA), 0.2 mM of each dNTP, 1.5 U of Dream Taq DNA polymerase (Thermo Scientific, USA) and 0.5 μl of 10 pmole (for COI) and 1.0 μl of 20 pmole (for 5S ISR and mt-lsr DNA) primers. PCR reaction was run for 35 cycles (5S ISR and COI) or 40 cycles (Mt-lsr DNA) each of 30 s at 95°C, 30 s at 55°C (for 5S ISR), 50°C (for Mt-lsr DNA) and 48°C (for COI) and 30 s at 72°C after the initial denaturation at 95°C for 5 min. A final extension of 10 min at 72°C was given for all three markers. The PCR products were visualized using 2% agarose gel electrophoresis at 5 V/cm for 40 min, and the gels were stained with RedSafe (iNtRON Biotechnology, Korea). The PCR product of each target gene was purified in bulk using QiAquick® gel extraction kit (Qiagen, Germany). Purified PCR products were finally submitted for nucleotide sequencing to the Eurofins Genomics (Bangaluru, India).

**Sequence analysis:** Nucleotide sequences for the 3 markers obtained by custom sequencing were analyzed using BLAST (blastn) programme (NCBI: http://blast.ncbi.nlm.nih.gov) and deposited in GenBankTM (Accession No. MH686440, MH686441 & MH686442). Representative sequences for 5S-ISR, Mt-lsr and COI gene were retrieved from NCBI database and used for comparative analysis (Table 2). Genetic diversity among the representative sequences of each selected marker was calculated using multiple alignments with Clustal W model in MegAlign module of DNASTAR (Laser gene Suite 6.0, USA). Phylogenetic analysis was performed using MEGA 7.0 software (Tamura et al., 2013), by the maximum likelihood method with best model fit (Tamura 3 parameter for 5S-ISR and Tamura Nei + G for both Mt-lsr and COI gene) using 1000 bootstrap replicates.

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**Table 1:** Primer sequences for selected marker genes.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5'- 3')</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5S rDNA-ISR</td>
<td>F- GCCGATTTGATCGACGACGGCCTG&lt;br&gt;R- GCCTCAGACGAGTGTGCCTTCAGC</td>
<td>750 bp</td>
<td>Liu et al. 1996; Rombout et al. 2001</td>
</tr>
<tr>
<td>Mt-lsr DNA</td>
<td>F- WACAAAGTCTCTTCTGTACT&lt;br&gt;R- TGAGGACATTAAAGTGAC</td>
<td>445 bp</td>
<td>Pozio et al. 2002</td>
</tr>
<tr>
<td>Cox-I gene</td>
<td>F- TACCTA ACTAAGAGGATTCGG&lt;br&gt;R- CTAGTACTCATAAGTGCTGTTG</td>
<td>750 bp</td>
<td>Franssen et al. 2015</td>
</tr>
</tbody>
</table>

**Table 2:** GenBank accessions included in the study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Accession Number</th>
<th>Species</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt-lsr DNA</td>
<td>GU357422, GU339147, GU339127, KM357414, KM357415, KM357413, KM357416, KM357417</td>
<td><em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. murrelli</em> (Tm), <em>T. nativa</em> (Tna), <em>T. britovi</em> (Tb), <em>T. spiralis</em> (Ts), <em>T. nelsoni</em> (Tne)</td>
<td>USA, China, China, USA, Norway, Italy, USA, Tanzania</td>
</tr>
<tr>
<td>Cox-I</td>
<td>GU357419, KM357422, MH119334, KU321696, MF668227, KM357414, KM357415, KM357416, KM357417</td>
<td><em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. spiralis</em> (Ts), <em>T. nativa</em> (Tna), <em>T. murrelli</em> (Tm), <em>T. nelsoni</em> (Tne)</td>
<td>USA, Poland, Belarus, Russia, Argentina, USA, Norway, Tanzania, Italy</td>
</tr>
</tbody>
</table>

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**Species identification study targeting different established markers viz. 5S ribosomal DNA intergenic spacer region (5S rDNA-ISR), partial mitochondrial large ribosomal subunit (Mt-lsr) gene and partial mitochondrial cytochrome c oxidase 1 (COI) gene.**
RESULTS AND DISCUSSION

Presence of nurse cells bearing Trichinella sp. larvae were recorded in muscle press preparation of masseter and diaphragmatic muscle under the microscope. Following acid-pepsin digestion, an average of 6 larvae per gram (lpg) was recorded for the different muscles examined. Larval burden was recorded as 10, 7, 5 and 2, respectively for the diaphragm, masseter, tongue and intercostal muscles.

Trichinella spp. infection in India has been recorded infrequently from different animal species and humans but diagnosis in each case has been made either by microscopic detection of muscle stage larvae or by histopathological examination of the biopsied material. Very recently, status of Trichinella sp. infection in India has been reviewed extensively and circulation of this parasite has been detected in 3 new host species viz. leopard, tiger and wild boar (Ram et al., 2018). However, in the light of current taxonomic status of 12 recognized species (9 species and 3 genotypes) of the parasite (Pozio and Zarlenga, 2013), species specific identification data from most of the Asian countries is not available. All the 12 genotypes also vary in host preferences and/or range, biological properties and geographical distribution and thus, correct identification of circulating Trichinella sp. from India is very much essential.

Present study is very unique and novel in context of India because of two reasons. Firstly, it describes T. spiralis infection in leopard (wild animal involvement) from a geographical area, in the vicinity of which outbreak of human trichinellosis had been recorded (Sethi et al., 2010). Secondly, for species level identification and establishing the phylogeny of circulating Trichinella sp., molecular biology tools were used for the first time. Till date, infection of T. nelsoni from Tanzania (Pozio et al., 1997) and T. britovi from Iran (Mowlavi et al., 2009) in leopards has been reported. A mixed infection of T. nelsoni and T8 genotype in leopard has also been reported from Greater Kruger National Park, South Africa (La Grange et al., 2014).

Multiplex PCR assay employed for the identification of Trichinella sp. in the given sample recorded single amplicon of 173 bp with CP-I set of primer (Fig 1). Amplification of 173 bp indicative of T. spiralis infection (Zarlenga et al., 1999), was found consistent with all 6 DNA samples (5 individual and 1 pooled larvae) used as template. The said primer has been found specific in amplification of the expansion segment five (ESV) region of T. spiralis in previous studies. Presence of this species in leopard indicates that infection is persisting in the forest area at the base of ecosystem i.e. in porcine population as well in the rodents.

PCR amplification of potential genetic markers viz. 5S-ISR, Mt-lsr rDNA and COI, yielded specific amplification of 750, 445 and 850 bp products, respectively in gel electrophoresis. Homology search analysis of 5S-ISR sequences generated in the study (MH686440), showed 99.6% sequence homology with T. spiralis sequences originating from China (KT894074). In phylogenetic analysis also, both T. spiralis isolates from China (KT894070, KT894074) and one isolate from USA (TSU65504) formed a cluster with Indian isolate. However, sequences of other Trichinella species or genotype included in the study from Netherlands (T. nativa-AO09944, T. murrelli-AO09947, Trichinella T8-AO09949), Argentina (T. patagoniensis-EF694983) and Italy (T. britovi-GU325734); formed a separate cluster (Fig 2). Similarly, homology search for the Mt-lsr sequences of T. spiralis Indian isolate (MH686441) showed 98.6% homology with the two T. spiralis isolates from China (GU339127, GU339147), while 98.4% similarity, each with T. spiralis isolates from Poland (KM357422) and USA (GU386314).

In phylogenetic analysis also, all 5 sequences of T. spiralis clustered together while sequences of other Trichinella species included in study formed a separate cluster (Fig 3). As like Mt-lsr sequence analysis, blast search analysis for
Fig 3: Mt-lsr based phylogeny of *T. spiralis* (Indian isolate).

Fig 4: COI gene based phylogeny of *T. spiralis* (Indian isolate).

COI sequence of Indian isolate (MH686441) showed more than 99.5% homology with *T. spiralis* sequences originating from Belarus (MH19334), Russia (KU321696), Poland (KM357422) and USA (GU386314). In phylogenetic analysis all *T. spiralis* isolates clustered together, while sequences of remaining species formed a separate cluster with variable sequence homology (Fig 4).

Specific PCR amplification of the selected genetic markers and sequences derived after custom sequencing further validated the results of multiplex PCR assay. Total length of nucleotides included for alignment were 698, 431 and 780 bp for the 5S ISR, Mt-lsr and COI gene, respectively. Amongst the selected markers, COI is supposed to give more appropriate differences in sequencing between different strains of the parasite compared to other genes used in previous studies viz, cyt B and 18S rDNA (Odoevskaya and Spiridonov, 2016). However, sometimes single marker shows a low variability between two or more taxa and does not constitute a suitable choice for species level identification (Marucci et al., 2010). Therefore, 5S ISR and Mt-lsr were also sequenced to confirm the results obtained in multiplex PCR. These selected markers have been found efficient in species level identification and phylogenetic analysis of all the 12 *Trichinella* genotypes (Zarlenga et al., 2006, Wang et al., 2012).

Homology search analyses of the selected markers revealed high level of sequence identity with the published sequences of *T. spiralis* and are in consent with previous studies (Wang et al., 2012; Bilska-Zajac et al., 2017). *T. spiralis* has been recorded very frequently from the bordering country of India i.e. China including Tibet, and it is possible that dispersal of infection might have occurred through the movement of wild animals across the border.

The phylogenetic analysis of 5S ISR, Mt-lsr and COI gene of *T. spiralis* (India) showed more robust correlations and clustered with published *T. spiralis* sequences in each case. At the same time, all other species or genotype of *Trichinella* included in the study clustered separately from *T. spiralis*. This shows the existing genetic variability amongst different species of *Trichinella* from the most primitive T1 genotype (*T. spiralis*). This is the first study from India, which provides molecular evidence on the circulation of *T. spiralis* in wild animals. Earlier, *T. pseudospiralis* has been described from India, but possibility of the presence of *T. spiralis* and *T. britovi* in country was also underlined (Pozio and Zarlenga, 2005).

In Uttarakhand state of India, there are large geographical areas under forests having rich heritage of wildlife including the elephant and tiger reserves. Also, there is under reported illegal hunting of wild animals taking place and poachers have the propensity of eating the undercooked and/ or smoked meat of wild animals which may result in outbreak in humans; as has been reported previously by Sethi et al. (2010). Meat of leopard or big cat is perhaps not the direct source of infection for human beings, but improper disposal of infected carcasses allows scavenging by other wild animals, especially omnivores like wild boars and rodents. There is all possibility that infection may spill over from the sylvatic cycle to the domestic animals and in such cases, public health will be at greater risk.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
REFERENCES


