MOLECULAR STUDIES REGARDING TO THE DIFFERENTIATION BETWEEN STRAINS OF *TRICHINELLA SPIRALIS* AND *T. PSEUDOSPIRALIS*

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Summary

*Trichinella* is an emerging and re-emerging causative agent of trichinellosis in humans, a food-originated zoonosis. In Europe it was found that domestic pigs are predominantly infected with three species of *Trichinella*, and at least two of these can coexist in the same animal. For adoption of correct epidemiological and therapeutic decisions, the discrimination and quantization of the infestation degree can be achieved only by molecular methods, since the tests based on morphological characters are not able to differentiate the isolates at the species level and to detect few larvae. For detection and discrimination between *Trichinella spiralis* and *Trichinella pseudospiralis* in this study we developed classic and real-time PCR methods, as single or multiplex, Sybr Green or Taqman reactions, based on hsp70 and shsp genes, respectively.

**Key words:** *Trichinella spiralis*, *Trichinella pseudospiralis*, PCR

Trichinellosis is a helminthic zoonosis widespread in the world, with significant implications for medical, veterinary and economical fields. It is produced by the nematode of the *Trichinella* genus, which is currently considered to be composed of eight species (*Trichinella spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murreli*, *T. nelsoni*, *T. Papuae*, *T. zimbabwensis*) and three additional genotypes (*Trichinella T₆*, related to *T. nativa* and *Trichinella T₅* and *T₆*, related to *T. Britovi*). *T. spiralis* and *T. pseudospiralis* have a cosmopolitan distribution, while other genotypes are restricted to certain zoogeographical areas (12). *Trichinella* species are considered as having an autoheteroxen biological cycle, because the adult parasites are found in host's intestine, and the larvae in migration or located into the muscles of the same individual (5). Various species of the genus *Trichinella* are distinguished between them by growing capacity, ability to induce the formation of a collagen capsule around the cell infected with the larval form of parasite, the ability to reproduce itself into a specific host, the number of larvae produced by females, enzyme profile, electrophoretic profile of larval proteins, sequence of heat shock proteins: Hsp70, and by genetic material sequence (9,16). For both humans and animals, the diagnostic of *Trichinella spp.* is done either by direct evidence of larvae in samples of muscle tissue (by trichinelloscopy or artificial digestion) or by serological evidence of parasite immunological fingerprint. Recent epidemiological data show that trichinellosis is a cosmopolitan parasite of carnivores, especially cannibals and necrophagous and it...
was identified in any investigated geographic area (10,15). Trichinellosis epidemiology is characterized by two main cycles: a synanthropic-domestic cycle (due to *Trichinella spiralis*) and a silvatic one (due to all species and phenotypes, including *Trichinella spiralis*) (5).

The most difficult part of the trichinellosis diagnostic, as in other parasitic infestations, is to realize the existence of the disease. The coprological examinations for identifying adults parasites are almost negative, because their number is small and they are often disintegrated (11). Occasionally, new-born larvae can be found in blood. The ability to detect the infestation could be significantly improved by applying of some gene amplification-PCR tests, for parasites detection (19).

In the last decade, molecular biology techniques have proved their applicability and usefulness in identification and differentiation of *Trichinella* species. The biomolecular identification methods tend to minimize the inherent subjectivity and duplicity of those methods based on biological and morphological features of the parasite (20,21). Nevertheless, the development and improvement of various techniques for genotypical identification of *Trichinella* species would not have been possible without the progress made to decipher the genome of this nematode.

In order to have a tool more appropriate destined to diagnostic and epidemiological survey of trichinellosis in animals, in this study we developed classic and real-time PCR methods for detection and discrimination between *Trichinella spiralis* and *Trichinella pseudospiralis*, as single or multiplex, Sybr Green or Taqman reactions, based on *hsp70* and *shsp* genes, respectively.

**Materials and methods**

*Trichinella spiralis* originates from Pasteur Institute, Bucharest, and *Trichinella pseudospiralis* (figure 1), from Community Reference Laboratory for Parasites, Italy. The larvae were prepared as is mentioned in CRLP documents (2, 3, 4).

![Fig. 1. Trichinella pseudospiralis (CRLP). Axiostar Microscope, Carl Zeiss (200x). Photo Canon Power Shot G3 (4x).](image-url)
DNA samples were obtained by the protocol previously published, with minor modification: 1-3 larvae were suspended in 2.5 ul Taq buffer (Stoffel, Perkin Elmer), with 1.5 ul MgCl2 25 mM and 20 ul ultra pure water, incubated at 90°C for 10 minutes, then treated with proteinase K (final concentration of 77 ug/ml) for 3h at 65°C (13). The primers for classical elective amplification or single or multiplex in real time amplification of the genes *hsp70* (*Trichinella spiralis*) and *shsp* (*Trichinella pseudospiralis*) were designed online (14) or by the use of Beacon Designer v7.5 software (BD, figure 2), based on the sequences registered in GenBank and commercially synthesized (Generi-Biotech, Czech Republic); the size of amplicons and the sequences of the primers and of Taqman probes are presented in table 1 (1, 6). The concentration of the primers in each type of reaction, the volume of the reaction and the cycling program applied on the thermal cycler PE GeneAmp 9600 PCR System (*Applied Biosystem*), on the thermal cycler iCycler (*Biorad*) or on the thermal cycler Mx3005P (*Strategene*), are shown in table 2. Classical PCR reactions were performed as single or multiplex variants. Real time PCR reactions were performed as single variant (with Sybr Green, or Taqman probes labelled with Fam and Hex as fluorophores, and Rox as reference dye) or multiplex variants, with Taqman probes. One of the variant of real time PCR was a combination between primers designed by Primer3 software and the Taqman probes designed by the use of BD software.

![Image](image.png)

Fig.2. Beacon Designer 7.5 (testing version), for primer and Taqman probe design: search for virtual validation of multiplex method built virtually.

**Table 1**

<table>
<thead>
<tr>
<th>No.</th>
<th>Agent / gene</th>
<th>Primer / Taqman probe 5'-3' Sequence</th>
<th>Ampli-con</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>T. spiralis</em> / <em>hsp70</em></td>
<td>Ts-hsp70-F: gac agc aga aga aag aat tga aag cac cca gga taa gga ga</td>
<td>152 (133)</td>
<td>This study AY046874</td>
</tr>
<tr>
<td>2.</td>
<td><em>T. spiralis</em> / <em>hsp70</em></td>
<td>Ts-hsp70-F2: aat gac cca aga cca cca ttc cat ctt ttc ttc agc atc c</td>
<td>547</td>
<td>This study AY046874</td>
</tr>
<tr>
<td>3.</td>
<td><em>T. spiralis</em> / <em>hsp70</em></td>
<td>BD7-Ts-hsp70-F3: gaa cca aat tgc cga cga tgc cag cca ttc cgg gac ttta tga atg</td>
<td>76 (99)</td>
<td>This study AY046874</td>
</tr>
</tbody>
</table>
Post-amplification control and validation of the real time reactions was achieved by horizontal gel-electrophoresis in TBE 1.5x, and the digitalization of the images was processed by Easy RH system and the software Image 2WinPC v5.0.1 (Herolab GmbH); the estimation of the size of the obtained amplicons was performed by the software UnScanIt (Silk Sci. Co. v5.1, 9). As negative control and for control of specificity of the primers we used DNA sample obtained from Toxoplasma gondii.

**Results and discussions**

The elective and multiplex amplifications results for genes *hsp70* and *shsp* of *Trichinella spiralis* and *T. pseudospiralis* respectively, are presented in figures 3 – 6 and table 2. All the amplifications formats were controlled by gel-electrophoresis and the estimated amplicons were confirmed, with exception of classical format performed with beads and pairs of primers designed with Primer3 software and the single reaction in real time variant with beads and primers and Taqman probes designed by BD software for *T. spiralis* (no Ct / no amplicon). The size of amplicons were 152 bp for *T. spiralis* and 182 bp for *T. pseudospiralis*, in case of primers designed with Primer3 software, and 76 bp and 100 bp respectively in case of primers designed with BD software. The threshold value (Ct) of single real time amplification variant with Sybr Green for *T. spiralis*, was 26.95. For the first pairs of primers designed with Primer3 in the real time format with Taqman probes the threshold values (Ct) were 12.13 and 15.18. In case of primers designed with BD software in single reaction formats with 2x Brilliant multiplex QPCR master mix reagent (Stratagene) the recorded threshold values were 28.02 and 43.06 respectively. In case of the same primers but in multiplex real time format reaction the threshold values were 10.92 and 8.94 respectively. In case of DNA sample originated from *Toxoplasma gondii* were not recorded products in any amplification of the formats tested.
Fig. 3. *Trichinella spiralis*. Real time PCR, Sybr Green variant, with first pair of primers. Amplification plots. Mx3005P (Stratagene).

Fig. 4. *Trichinella spiralis*. Real time PCR, Sybr Green variant, with first pair of primers. Dissociation curve. Mx3005P (Stratagene).

Fig. 5. *Trichinella spiralis* (IP) and *Trichinella pseudospiralis* (CRLP). Real time PCR and multiplex real time PCR with Taqman probes. Amplification plots. Mx3005P (Stratagene).
Fig. 6. *Trichinella spiralis* (IP) and *Trichinella pseudospiralis* (CRLP). Real time PCR and multiplex real time PCR with Taqman probes. Control by gel-electrophoresis TBE 1.5x in order to validate the amplification in real-time variant.

### Table 2

Cycling programs used during this study

<table>
<thead>
<tr>
<th>Primer / Taqman probe</th>
<th>Primers concentration (pmole)</th>
<th>Thermal cycler / Program</th>
<th>Volume reaction / mix</th>
<th>Ampli- con / Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR</td>
<td>mPCR</td>
<td>Q PCR</td>
<td>mQ PCR</td>
</tr>
<tr>
<td><strong>Ts-hsp70-F</strong></td>
<td>30</td>
<td>-</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Ts-hsp70-R</strong></td>
<td>50</td>
<td>12.5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td><strong>Ts-hsp70-F2</strong></td>
<td>50</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Ts-hsp70-R2</strong></td>
<td>50</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>BD7-Ts-hsp70-F3</strong></td>
<td>-</td>
<td>-</td>
<td>12.5</td>
<td>5</td>
</tr>
</tbody>
</table>

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To our knowledge the multiplex real-time PCR variants developed in this study are the first assays detecting and distinguishing *T. spiralis* and *T. pseudospiralis* by means of Taqman probes (7,17,18).

Conclusions

The data of molecular investigations obtained during this study concluded to the following:

1. Primers designed by the use of the two softwares proved to be specific for *Trichinella spiralis* and for *T. pseudospiralis*, respectively.

2. All the amplification variants, cycling programs and reaction mixes, had positive results, with three exceptions.
3. The multiplex variant proved to be faster and discriminated between the two species of *Trichinella* under study, and can be used in further studies to develop new tools destined to diagnostic and epidemiological survey of trichinellosis.

**Acknowledgements**

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**References**

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