A SINGLE, MULTIPLEX PCR FOR DIFFERENTIATING ALL SPECIES OF *TRICHINELLA*

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Summary :

The genus *Trichinella* is currently divided into seven species and at least three additional, unclassified genotypes, *Trichinella* T6, T8 and T9, where both T8 and T9 have been deemed very similar to *T. britovi*. Other than for the non-encapsulated species, the absence of distinguishing morphological characters and the overlapping nature of the biological characters within this genus make these traits unsuitable for diagnosis. Consequently, we have developed a simple PCR test for the unequivocal differentiation of all currently recognized species of *Trichinella* including *Trichinella* T6. DNA sequence data from each *Trichinella* genotype were generated from internal transcribed spacers, ITS1 and ITS2, and from expansion segment V (ESV) of the rDNA repeat, from which five different PCR primer sets were chosen. When used simultaneously, this primer mix generates a simple and unique electrophoretic DNA banding pattern for each species and genotype. The ESV-derived primer set contributes at least one band to each agarose gel-derived genotypic pattern and therefore functions as an internal control for PCR integrity. Geographical isolates of each *Trichinella* genotype were used to verify the reliability and reproducibility of respective DNA banding patterns using single muscle larvae.

KEY WORDS : diagnosis, PCR, multiplex, ribosomal DNA.

Within most parasite genera, distinct morphological and/or biological characters exist among the species that permit differentiation and classification. However, other than for non-encapsulated species, the absence of distinguishing morphological characters and the overlapping nature of the biological characters within the genus *Trichinella* make these traits unsuitable for accurate diagnosis. In 1988 the International Commission on Trichinellosis established the *Trichinella* Reference Center (TRC) (Pozio *et al.*, 1989) at the Istituto Superiore di Sanita' in Rome, Italy, and empowered it to function as an international resource for identifying and cryopreserving *Trichinella* parasites. Since that time, and for

some years prior, the methods used by the TRC for *Trichinella* differentiation have been based primarily on biochemical and molecular characters, though multifaceted approaches have been invoked for isolates of greater interest.

Currently, the most reliable methods for *Trichinella* differentiation are based upon PCR for its ease of use, sensitivity and objectivity in differentiating genotypes. Numerous researchers have utilized both random amplified polymorphic DNA (RAPD) primers (Arribas *et al.*, 1994; Bandi *et al.*, 1993; Dupouy-Camet *et al.*, 1994) as well as specific primers (Wu *et al.*, 1997; Wu *et al.*, 1998) to achieve this goal. The major drawback of RAPD-derived techniques has been the lack of specificity and reproducibility whereby a complete bank of positive controls must be run with each analysis. The use of species/genotype specific primers, though preferable, can require a substantial amount of trial and error for proper identification. Some have tried to minimize these problems by secondary treatment of PCR products with restriction enzymes (PCR-RFLP) for final differentiation (Wu *et al.*, 1999); however, since multiple steps are required, this potentially can lead to incorrect diagnosis should extraneous factors effect subsequent reactions and data interpretation. Zarlenga *et al.* (1999) recently developed a multiplex PCR test based upon sequence length polymorphism within the ESV region and sequence variability within the ITS that simplifies *Trichinella* differentiation into a single step PCR. Herein we summarize the development of this test and demonstrate its applicability in diagnosing the newly characterized *T. papuae*.

MATERIALS AND METHODS

Trichinella genotypes were maintained by serial passage in Swiss-Webster female mice. Infective larvae were recovered from ground muscle tissue by pepsin-HCl digestion from which genomic DNA was extracted using proteinase K:SDS digestion (Zarlenga *et al.*, 1999).

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Primer pair	Ref. No.	Location on DNA	Sequence
I	25(5')* 268(3')*	ESV	5'GTTCCATGTAACAGCAGT 5'CGAAAACATACGACAACCTGC
II	553(5') 545(3')	ITS1	5'GCTACATCCTTTTGATCTGTT 5'AGACACAATATCAACCACAGTACA
III	583(5') 564(3')	ITS1	5'GCGGAAGGATCATTATCGTGTA 5'TGGATTACAAAGAAAACCATCACT
IV	495(5') 496(3')	ITS2	5'GTGAGCGTAATAAAGGTGCAG 5'TTCATCACACATCTTCCACTA
V	568(5') 536(3')	ITS2	5'CAATTGAAAACCGCTTAGCGTGTTT 5'TGATCTGAGGTCGACATTTC

5' = primer at 5' end of amplified sequence
3' = primer at 3' end of amplified sequence

Table I. – Primer sequences for multiplex PCR.

Genomic DNA (1 ng) from each *Trichinella* genotype was amplified in a multiplex PCR reaction containing primer pairs I, II, III, IV and V (Table I). Primer sets were generated from sequence alignments of both the ESV and ITS regions of the rDNA repeat from each *Trichinella* genotype. All primer concentrations were 0.25 M except for primer pair IV (Table I) which was used at 0.5 M. Reactions were performed in 50 µl of Ex-Taq PCR buffer supplemented with 0.2 mM each dNTP, 2.5 U Ex-Taq DNA polymerase (Panvera Corporation) and 1 % v/v dimethyl sulfoxide. All reactions were carried out in a Perkin-Elmer 480 Thermal Cycler under the following cycling conditions: 94°C, 1 min; 60°C, 1 min; 72°C, 2 min, for 40 cycles. Reaction products (5 µl) were separated on a 2.5 % Metaphor agarose gel and detected by ethidium bromide staining.

M 1 2 3 4 5 6 7 8 9 10

Fig. 1. – Agarose gel analysis of *Trichinella* multiplex PCR. Polymerase chain reaction was performed on reference strains of *Trichinella* and the PCR products were separated on a 2.5 % Metaphor agarose gel followed by ethidium bromide staining. 1) *T. spiralis*; 2) *T. nativa*; 3) *T. britovi*; 4) *T. pseudospiralis* (Russia); 5) *T. pseudospiralis* (USA); 6) *T. pseudospiralis* (Tasmania); 7) *T. murrelli*; 8) *Trichinella* T6; 9) *T. nelsoni*; 10) *T. papuae*.

RESULTS

Results from agarose gel electrophoresis of multiplex PCR products using genomic DNA from reference strains of the different genotypes of *Trichinella* are shown in Fig. 1. The approximate size of each PCR fragment and the corresponding primer set(s) responsible for generating each fragment are summarized in Table II. Results indicate a unique and simple banding pattern for each genotype including the newly described *T. papuae*. At least one PCR fragment from each genotypic banding pattern was generated from the lsrDNA- derived ESV. Banding profiles were consistent among geographical isolates of similar genotype (data not shown).

Primers ^a	Locus ^b	Trichinella genotype and DNA Fragment size ^c									
		T1	T2	T3	T4 ^r ^d	T4 ^t ^d	T4 ^u ^d	T5	T6	T7	T10
I	ESV	173	127	127	310 ^e	340 ^e	360 ^e	127	127	155	248
II	ITS1			253							
III	ITS1							210			
IV	ITS2						316				
V	ITS2							404			

^a Primer sets as reported in Table I.

^b Locus on genome where primers anneal; ESV = expansion segment V; ITS = internal transcribed spacer.

^c All sizes are approximate and are given in base pairs.

^d T4 isolates from Russian (r), Tasmania (t) and the USA (u).

^e Size listed involves a cluster of 2 or more fragments.

T1 = *T. spiralis*; T2 = *T. nativa*; T3 = *T. britovi*; T4 = *T. pseudospiralis*; T5 = *T. murrelli*; T7 = *T. nelsoni*; T10 = *T. papuae*.

Table II. – DNA fragment sizes generated from multiplex PCR.

DISCUSSION

Until now, a simple, one-step assay for the unilateral differentiation of all *Trichinella* genotypes has not been available. The multiplex PCR described herein exploits the size variability within the ESV to differentiate *T. spiralis*, *T. pseudospiralis*, *T. papuae* and *T. nelsoni* species from all other sylvatic species and genotypes. Because the ESV-derived primer set is conserved among all *Trichinella* genotypes studied thus far, its PCR products represent diagnostic characters as well as internal positive controls for the PCR.

Partial analysis and alignment of the ITS1 and ITS2 sequences has provided the basis upon which additional PCR primers were developed for distinguishing *T. britovi* and *T. murrelli*. A third primer set differentiates *T. nativa* from *Trichinella* T6, which is also freeze resistant and genetically similar to *T. nativa*. In so doing, this primer set establishes the lone appearance of the 127 bp ESV-derived fragment common to most sylvatic genotypes, as a specific diagnostic character for *T. nativa*.

The reliability and reproducibility of each DNA banding pattern was verified with single muscle larvae using geographical isolates of each *Trichinella* genotype (data not shown). PCR amplification profiles were conserved among multiple geographical isolates representing each genotype tested, as well as individual parasites within a discrete population (data not shown). The origins of the isolates analyzed were sufficiently diverse to support the claim that the banding patterns presented here will persist as reference profiles for each genotype; however, final validation of the technique prior to routine application will require analysis of more isolates and standardization. To this end, the International Trichinella Reference Center has taken a leadership role in further validating the PCR assay and to date has successfully identified 92 isolates of *T. spiralis*; 84 isolates of *T. nativa*; 95 isolates of *T. britovi*; 10 isolates of *T. pseudospiralis*; 12 isolates of *T. murrelli*; 11 isolates of T6; 2 isolates of *T. nelsoni*; and 1 isolate of *T. papuae*.

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