ESTIMATING THE GENETIC DIVERGENCE AND IDENTIFICATION OF THREE *TRICHINELLA* SPECIES BY ISOENZYME ANALYSIS

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Summary :

Isoenzyme-based approach was applied to compare *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis* species. Among 13 enzyme systems examined, esterase (EST), malic enzyme (ME) and phosphoglucumutase (PGM) have been found as fully diagnostic, with no common allele in species studied. Adenosine deaminase (ADA), adenylate kinase (AK), hexokinase (HK), peptidase leucyl-alanine (PEP-C) and fructose-bis-phosphatase (FBP) have been capable of distinguishing the two species from resulting profiles. In addition, ADA, AK and PGM displayed the enzyme expression in the lowest amounts of muscle larvae in systems tested (100 larvae/100 µl of extracts). Based on allozyme data, *T. pseudospiralis* has been found as the most distinct species within the group of taxa. Only a subtle genetic variability was recorded for *T. pseudospiralis* in which solely phosphoglucumutase exhibited variant patterns. In addition to the study of reference isolates, *T. spiralis* from lowland fox in Eastern Slovakia has been evidenced by use of genetic markers. This finding has proved that *T. britovi* is not the exclusive species parasitizing in the sylvatic ecosystem of the Slovak region.

KEY WORDS : *Trichinella*, isoenzymes, diagnosis, isoelectrofocusing.

The genus *Trichinella* is characteristic of the widespread geographical distribution and the large range of host species. Until recent years, *Trichinella* parasites have been characterized mainly by the behavioural features as host specificity, tissue distribution of larvae, infectivity, reproductive capacity, resistance to lower temperatures, i.e. With the possible exception of *T. pseudospiralis*, there are no clear-cut morphological differences between *Trichinella* isolates. In addition, high variability in most of biological characters that can be influenced by selective pressure of different host/environment has not allowed relevant taxonomic conclusions.

Molecular and biochemical studies afford a useful approach for delineating *Trichinella* taxa (Koman-darev *et al.*, 1986; Turčėková *et al.*, 1997; Appleyard

et al., 1999), and highly contributed to the taxonomic stability of the genus that has been revised particularly by Pozio *et al.* (1992a). Isoenzyme approach provides an effective tool for delineating closely related organisms and has been used in more studies elucidating polymorphism in the *Trichinella* genus (Mydinski & Dick, 1985; Fukumoto *et al.*, 1988; Snyder *et al.*, 1993). Enzymatic profiles generated by electrophoresis can be achieved by the isoelectric focusing technique which separates proteins which differ in their isoelectric point (i.e. the pH where the net charge of a protein is electrically neutral).

The current study was undertaken to provide consistent characters for differentiating three important *Trichinella* species using the above technique, to assess the degree of genetic similarity among them and to study the enzyme expression in various quantities of pools of muscle larvae.

MATERIAL AND METHODS

Isolates of *Trichinella spiralis* (T1) from domestic pig (USA), code ISS004; *T. spiralis* (T1) from red fox (Slovakia), code ISS632; *T. britovi* from wolf (Italy), code ISS100; *T. pseudospiralis* from raccoon dog (Caucasus, Russia) code ISS013; *T. pseudospiralis* from black vulture (USA), code ISS470; *T. pseudospiralis* from tiger cat (Australia) and *T. pseudospiralis* (Caucasus, Russia) from raccoon have been involved in this study.

From these, Slovak isolate of *T. spiralis* and from wild-life population and *T. pseudospiralis* from raccoon has been since recovery passaged in SPF mice of ICR strain. Remaining isolates are those of reference isolates from the *Trichinella* Reference Centre in Rome. Following experimental infection of rats (performed on inbred Fisher 344 strain) on reference samples, three above species from nine taxa have been adequately amplified, thus providing sufficient amount of muscle larvae for subsequent allozyme analyses. Minced carcasses were digested in an artificial gastric juice, larvae were concentrated by sedimentation and washed several times in 0.85 % saline. Samples for electro-

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phoresis have been prepared by crushing the parasites in the enzyme stabilizer solution (1 mM EDTA, and 1 mM dithiothreitol in Tris-HCl; pH 7.4).

Isoelectrofocusing technique has been carried out in thin layers of polyacrylamide gels. For the establishment of a pH gradient, 3 ml of carrier ampholytes of the pH ranges 3.5-9.5 and 4.0-6.5 were added to the gel solution. After the completion of electrophoresis, gels were stained at 37°C until fully developed.

The factorial analysis of correspondence (Lebart, 1984) was used to screen genetic relationships among taxa studied. This method characterized each gene pool simultaneously according to all variables (i.e. genetically encoded isoenzymes. To evaluate the degree of similarity between paired genotypes, Jaccard's coefficient based on a number of shared isoenzymes and a number of mismatches was calculated.

RESULTS

The following 13 enzyme systems have been clearly resolved: adenosine deaminase (ADA, EC 3.5.4.4); adenylate kinase (AK, EC 2.7.4.3); esterase (EST, EC 3.1.1.1); fructose-bisphosphatase (FBP, EC 3.1.3.11); hexokinase (HK, EC 2.7.1.1); lactate dehydrogenase (LDH, EC 1.1.1.27); malate dehydrogenase (MDH, EC 1.1.1.37); malic enzyme (ME, EC 1.1.1.40); mannose-phosphate isomerase (MPI, EC 5.3.1.9); peptidase leucyl-glycine-glycine (PEP-B, EC 3.4.11.4); peptidase leucyl-alanine (PEP-C 3.4.13.11); phosphoglucomutase (PGM, EC 2.7.5.1) and superoxide dismutase (SOD, EC 1.15.1.1).

Among enzymes examined, EST, ME and PGM were capable to delineate *T. spiralis*, *T. britovi* and *T. pseudospiralis* spp. in expressing fixed allelic differences between all species. In addition, isoenzyme patterns obtained from ADA, AK, HK, PEP-C, FBP were found partially diagnostic, with one taxon within the three being distinguished (Table I). On the other hand, identical profiles were displayed when analysing MDH, SOD, and PEP-B enzyme activities. LDH has not been resolved in the sufficient activity for T2, while same patterns were found out in T1 and T3 for this enzyme. MPI gave two different signals in T1 and T3, with both isoenzymes presented in T4 isolates.

In studying the genetic variability in four isolates of *T. pseudospiralis*, visible bands were recorded for all samples with eight enzymes (ADA, EST, LDH, MDH, PEP-B, PGM, SOD and ME). Only in PGM were found out polymorphic patterns. Isolates recovered from Australia and Caucasus (host - raccoon) displayed one common allele with those obtained from Caucasus (host - raccoon dog) and Australia, whereas the second allele exhibited the slower electrophoretic mobility in

PGM

Fig. 1. – Isozyme patterns of phosphoglucomutase. Lane 1, *T. pseudospiralis* (Caucasus, raccoon dog); lane 2, *T. pseudospiralis* (Australia, tiger cat); lane 3, *T. pseudospiralis* (Caucasus, raccoon); lane 4, *T. spiralis* (USA, pig); lane 5, *T. spiralis* (Slovakia, red fox).

ADA

Fig. 2. – Isozyme patterns of adenosine deaminase. Lane 1, *T. pseudospiralis* (Caucasus, raccoon dog); lane 2, *T. pseudospiralis* (USA, black vulture); lane 3, *T. pseudospiralis* (Australia, tiger cat); lane 4, *T. spiralis* (USA, pig); lane 5, *T. britovi* (Italy, wolf).

EST

Fig. 3. – Isozyme patterns of esterase. Lane 1, *T. pseudospiralis* (Caucasus, raccoon dog); lane 2, *T. pseudospiralis* (USA, black vulture); lane 3, *T. pseudospiralis* (Australia, tiger cat); lane 4, *T. spiralis* (USA, pig); lane 5, *T. britovi* (Italy, wolf).

the former samples (Fig. 1). Isoenzymes detected in all systems are in Table I. Photographs of diagnostic enzymes ADA and EST are given in Fig. 2 and 3.

Enzyme	T1	T3	T4
PGM	95	104	98, 100, 101
EST	94	85	100
ME	106	109	100
ADA	100	100	110
HK	100	108	100
AK	100	106	100
MPI	97	100	97, 100
PEP-B	100	100	100
PEP-C	100	100	104
FBP	100	100	98
LDH	100	–	100
MDH	100	100	100
SOD	100	100	100

T1 : *Trichinella spiralis*.

T2 : *Trichinella britovi*.

T4 : *Trichinella pseudospiralis*.

Table I. – Isoenzymes detected in 3 *Trichinella* species.

Jaccard's index of similarities gave the values of 0.33 between T1 and T3, 0.18 when comparing T3 and T4, and 0.27 for T1 and T4. Based on isoenzyme data, factorial analysis of correspondence identified *T. pseudospiralis* as the most diverged taxon within species concerned (Fig. 4).

In estimating the minimal amounts of larval quantities that allow manifestation of enzyme activity, ADA and AK have been found as most suitable enzymes for these purposes. Both enzymes showed visible bands from the concentration of 100 larvae/100 µl in homogenizing solution (amounts of substrates used: 80 mg of adenosine for ADA and 120 mg of glucose for AK, respectively, in the 40 ml volume of staining buffer). This was also the case of PGM, however, with this system the activity of enzyme decreased substantially in homogenates being stored for longer periods at 4°C.

DISCUSSION

The present study demonstrates that esterase, malic enzyme and phosphoglucosmutase may function as markers for differentiating three *Trichinella* genotypes under study. When assessing genetic relationships among taxa, *T. pseudospiralis* has been found as the most diverged species within this group. These data are in accordance with the view of Britov (1985) who regarded this taxon as the oldest species in the genus, adapted to many hosts, with the lack of capsule formation in the musculature. Accordingly, RAPD dendrograms in the study of Bandi *et al.* (1995) have positioned *T. pseudospiralis* into the sister group to other (cyst-producing) *Trichinella* taxa.

Only a moderate level of genetic variability was recorded in *T. pseudospiralis* coming from different zoogeographical regions (Palearctic and Australian regions) as well as mammalian and avian hosts. These data are corresponding with those obtained by Pozio *et al.* (1992b) in enzyme electrophoresis on starch gels. On the other hand, Zarlenga *et al.* (1996) reported remarkable population variation in this species in microsatellite sequences. However, microsatellite markers are recognised as much more polymorphic in comparison with loci encoding enzymes in which functional constraints generally prevent higher expression of polymorphism (Grant, 1994). Interestingly, the only enzyme exhibiting variation in *T. pseudospiralis* (PGM) displayed differences in the two Russian isolates despite the similar former hosts and regions involved. However, given that both isolates had been subjected to many passages since its isolation (recovery in 1972), there has been a high probability of occurrence of genetic drift in samples considered.

Fig. 4. – Horizontal axis explaining 58 % of the total variance, vertical axis explaining 42 % of the total variance. T₄ was placed in the most distinct position along axis 1.

Pools of muscle larvae analysed with isoenzyme-based approach excluded the evaluation of allele and genotype frequencies. Thus, zymodeme approach as described by Andrews & Chilton (1999) has been applied for our samples, with the assessment of entire allozyme pattern. Presumed secondary isozymes resulting from post-translational modifications have not been involved into the statistical estimations. Apart from drawback of this method in requiring relatively large amount of samples, the main advantages are those of high degree of reproducibility and no need for high sample purification, unlike the RFLP-DNA analysis (Wang *et al.*, 1995). In comparison with the most complex isoenzyme study of *Trichinella* till now performed by La Rosa *et al.* (1992a), hexokinase and fructose-bis-phosphatase enzymes have been supplemented in the present study. Field isolate collected in 1998 in the Eastern Slovakia from the lowland fox has been identified as *T. spiralis*. In addition to the predominant *T. britovi* species in the Slovak region, a less frequent *T. spiralis* can thus also sporadically occur in sylvatic animals, despite that no infection of its principal host (domestic pig) has been recorded in last years. Molecular studies might assist in assessing the risk for men and the better knowledge of routes of infection in territories of interest.

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