EVALUATION OF TWO PCR-BASED TECHNIQUES FOR MOLECULAR EPIDEMIOLOGY IN FINLAND, A HIGH-ENDEMIC AREA WITH FOUR SYMPATRIC *TRICHINELLA* SPECIES

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Summary :

Trichinella larvae collected from wildlife, domestic and synanthropic animals in Finland were identified to species by two molecular techniques: Random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) and the recently described multiplex PCR. The RAPD-PCR was very sensitive to the sub-optimal preservation muscle larvae and resulting in weak and smeared bands on the gels for such material. However, the same samples yielded easily recognizable bands in the multiplex PCR; this latter technique is then recommended for epidemiological studies, especially when the preservation of the samples is sub-optimal. For larvae in good condition the unequivocal bands obtained by multiplex was the easiest identifiable. Four species of *Trichinella* were identified in the material: *T. spiralis, T. nativa, T. britovi,* and *T. pseudospiralis*. *Trichinella britovi* is a new record for Finland, and *T. pseudospiralis* is a new record for Northern Europe. Mixed infections between *T. britovi* and *T. spiralis, T. nativa* and *T. spiralis,* and between *T. britovi* and *T. nativa* were detected; this is the first record of a mixed infection between *T. spiralis* and *T. nativa* in a naturally infected host. Raccoon dogs were the only host species from which all of the four *Trichinella* species were detected. *Trichinella spiralis* was found in both domestic animals and wildlife, but none of the sylvatic *Trichinella* species were detected in domestic pig.

KEY WORDS : RAPD-PCR, multiplex PCR, epidemiology, *Trichinella spiralis, Trichinella nativa, Trichinella britovi, Trichinella pseudospiralis.*

Up to now, species determination of *Trichinella* spp. in epidemiological studies have mainly employed random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) analysis (Bandi *et al.*, 1995), but the reproducibility of the RAPD-PCR has been questioned because the technique is relatively sensitive to sub-optimally preserved samples (Pozio *et al.*, 1999). Similarly, it has been demonstrated from experimentally infected pigs that sylvatic species of *Trichinella* may be difficult to identify by RAPD-PCR, presumably due to incomplete development in the pig muscle tissue (Pozio *et al.*, 1999). Recently, Zarlenga *et al.* (1999) introduced a less equivocal technique for simultaneous differentiation of all genotypes of *Trichinella.*

It is evident from previous studies that Southern Finland is a high endemic area for sylvatic and domestic trichinellosis (Hirvelä-Koski *et al.*, 1985; Oivanen & Oksanen, 1994; Mikkonen *et al.*, 1997; Oksanen *et al.*, 1998), although none of the studies have used molecular techniques for differentiation of *Trichinella* species in the Finnish wildlife and domestic animals. Because of the exceptionally high regional prevalence in wildlife (Oivanen & Oksanen, 1994; Oksanen *et al.*, 1998) and recurrent infections in domestic swine (Oivanen & Oksanen, 1994), southern Finland presents an ideal opportunity for studies on the molecular epidemiology of *Trichinella.*

The aims of the present study were to evaluate two techniques for molecular epidemiology in an area with sympatric species of *Trichinella.* It is the first time this multiplex PCR technique is used for an epidemiological study.

MATERIALS AND METHODS

Muscle samples were collected in Southern Finland (1993-1997) from nine host species (bears, wolves, foxes, lynx, raccoon dogs, wild boars, domestic pigs, rats and cats). After initial examination, 87 muscle samples positive for *Trichinella* were stored at – 20° C. However, all samples from rats were used for other purposes prior to the present study and were therefore thawed twice.

Muscle larvae were released by HCl pepsin digestion and recovered larvae were stored in 70 % ethyl-alcohol and stored at – 20° C. Prior to molecular analysis, larvae were rehydrated in a series of alcohol and single larvae were finally placed in 5 µl sterile water. The condition of the larvae, was determined from the appearance of the larvae. Coiled and partly uncoiled larvae were described as in moderate condition and

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C-shaped larvae with a transparent appearance as in bad condition.

Molecular identification was done as a blind study in two laboratories each with one PCR-derived techniques: Random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) was done at the Danish Centre for Experimental Parasitology and multiplex PCR at the International *Trichinella* Reference Centre (ITRC). For each muscle isolate, three or more larvae were tested by each of the methods, except for a few samples where fewer larvae were available; in these instances all larvae available were tested.

The RAPD-PCR was done according to Bandi *et al.* (1995) using primer 494 (AGCGCTGTGAGAAAGATGAAAGAT) at the Danish Centre for Experimental Parasitology. Larvae of reference strains *T. spiralis* (code ISS4), *T. nativa* (ISS42), and *T. britovi* (ISS100) were included in each reaction and resulting gel. Initially, *T. pseudospiralis* was not expected to be found in Finland and was not included as a reference in the RAPD-PCR.

The multiplex PCR was modified from Zarlenga *et al.* (1999) according to a protocol developed at the ITRC. Briefly, a solution of 0.1 M Tris-HCl pH 7.6 was added to the larva, overlaid with mineral oil, heated at 90°C for 10 min, treated with proteinase K (final concentration: 10 mg/ml) at 55° C for three hours, and heated again at 90° C for 10 min. PCR was performed using *Taq* DNA polymerase, 10 X PCR buffer, and dNTPs, from Takara (Japan), preparing 50 µl containing a final concentration of 1.5 mM $MgCl_2$ (included in the Takara 10X PCR buffer), 0.2 mM dNTPs, 0.5 unit of *Taq* DNA polymerase, and 50 pmoles of each of five primer pairs (I: 5´-GTTCCATGTGAACAGCAGT + 5´-CGAAAACATACGACAACTGC, II: 5´-GCTACATCCTTTTGATCTGTT + 5´-AGACACAATATCAACCACAGTACA, III: 5´-GCGGAAGGATCATTATCGTGTA + 5´-TGGATTACAAAGAAAACCATCACT, IV: 5´-GTGAGCGTAATAAAGGTGCAG + 5´-TTCATCACACATCTTCCACTA, V: 5´-CAATTGAAAACCGCTTAGCGTGTTT + 5´-TGATCTGAGGTCGACATTTCC).

For amplification, 4 µl of 10 µl of a single larva preparation were used according to Pozio *et al.* (1999). Amplifications consisted of 35 cycles, as follows: denaturation at 94° C for 20 s, annealing at 58° C for one minute, and elongation at 72° C for one minute. For the comparison, ML of reference strains for *T. spiralis* (code ISS3), *T. nativa* (ISS10), *T. britovi* (ISS2), and *T. pseudospiralis* (ISS13), were used.

The kappa coefficient (K) was used to describe and to test the degree of agreement between RAPD and multiplex species identification. Fishers exact test was used to test if RAPD-PCR was influenced by the preservation of the larvae.

RESULTS

An overall agreement was obtained for species identification of *Trichinella* larvae with both the RAPD-PCR and the multiplex PCR (K = 0.691, SE 0.064, $p < 0.001$, proportion of matches (agreements) 69/87 = 0.793, proportion of matches expected by chance = 0.330). However, it was obvious that the RAPD-PCR was very sensitive to the physical condition of the *Trichinella* larvae (Fishers Exact test, $P < 0.001$), and that bands of *T. spiralis* and *T. britovi* were difficult to distinguish from each other in the RAPD-PCR (Tables I and II). In contrast, the simple band patterns produced by the multiplex PCR allowed for faster and less subjective identification of species specific bands. Larvae judged as in bad condition (uncoiled and transparent) gave weak and smeared bands in the RAPD-PCR (Fig. 1). With the same samples, the multiplex PCR produced bands for which both molecular weight and

	Bear		Cat		Fox		Lynx		Racoon dog		Rat		Wild boar		Wolf		Pig		Total	
	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M
T. spiralis			1*	2	1	1			6	5	16	28	2	2			9	10	35	48
T. nativa	3	3			10	10	3	3	11	11					1	1			28	28
T. britovi																			0	0
T. pseudospiralis									4	4		1	1	1					5	6
T. spiralis + *T. britovi*			1						1	2									2	2
T. spiralis + *T. nativa*					1	1			1						1				3	1
T. nativa + *T. britovi*										1						1			0	2
Unclear bands											13						1		14**	0
Total																			87	87

R: RAPD-PCR (Bandi *et al.*, 1995).
M: Multiplex PCR (Zarlenga *et al.*, 1999).
*: Number of positive samples.
**: All frozen and thawed twice: *Trichinella* larvae in bad condition (Uncoiled or transparent).

Table I. – Species identification of *Trichinella* from nine host species by two PCR methods.

		Multiplex PCR								
		T. spiralis	*T. nativa*	*T. britovi*	*T. pseudo-spiralis*	*T. spiralis* + *T. britovi*	*T. spiralis* + *T. nativa*	*T. nativa* + *T. britovi*	Unclear bands	Total
RAPD-PCR	*T. spiralis*	34				1				35
	T. nativa		28							28
	T. britovi			0						0
	T. pseudospiralis				5					5
	T. spiralis + *T. britovi*	1				1				2
	T. spiralis + *T. nativa*						1	2		3
	T. nativa + *T. britovi*									0
	Unclear bands	13			1					14
	Total	48	28	0	0	2	1	2	0	87

Table II. – Comparison of test results of 87 individual muscle samples.

similarity to the reference strains were easy to interpret (Fig. 2). Highest discrepancy between the methods were often found in the differentiation of *T. spiralis* from *T. britovi* and visa versa. Except for badly preserved larvae, there were no significant differences between the two methods for differentiating *T. pseudospiralis* and *T. nativa*.

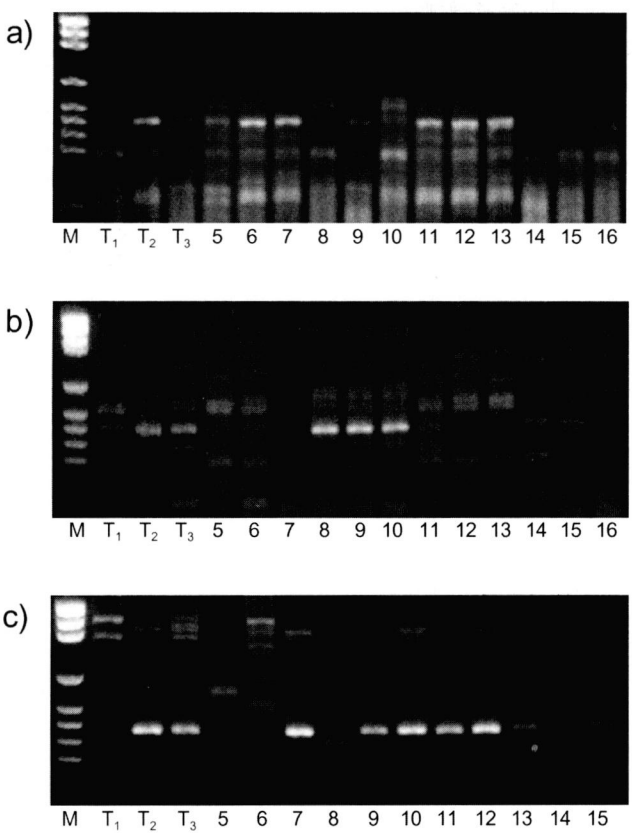

Fig. 1. – a-c: Examples of RAPD-PCR patterns obtained with crude preparations of individual *Trichinella* muscle larvae. Lanes: M: 100 bp ladder (PGEM); Reference larvae: T1, *Trichinella spiralis* (ISS4), T2: *Trichinella nativa* (ISS42), T3: *Trichinella britovi* (ISS100).
a: Lanes: 5-7 (wolf): 1x *T. spiralis*, 2x *T. nativa*, 8-10 (cat): 1x *T. britovi*, 2x *T. spiralis*,11-13 (raccoon dog): 3x *T. nativa*,14-16 (raccoon dog): 3x *T. spiralis*.
b: Lanes: 5-7 (pig): 2x *T. spiralis* (7: no product), 8-10 (lynx): 3x *T. nativa*,11-13 (pig): 3x *T. spiralis*, 14-16 (wild boar): 3x *T. pseudospiralis*.
c: Lanes: 5-7 (fox): 1x *T. spiralis*, 1x *T. nativa* (5: unclear), 8-10 (fox): 2x *T. nativa* (8: unclear), 11-13 (bear): 3x *T. nativa*, 14+15 (rat): unclear (C-shaped and transparent larvae).

Four species of *Trichinella* were identified from the nine host species: *T. spiralis*, *T. nativa*, *T. britovi*, and *T. pseudospiralis* (Table I). Raccoon dogs were the only host species from which all of four *Trichinella* species were identified. Foxes and wolves had *T. spiralis* and *T. nativa*, cats *T. spiralis* and *T. britovi*, rats and wild boars *T. spiralis* and *T. pseudospiralis*, pigs only *T. spiralis*, and lynx and bear only *T. nativa*. Mixed infections were found in raccoon dogs, foxes, wolves, and cats. None of the mixed infections contained more than two *Trichinella* species (*T. spiralis* + *T. britovi*, *T. spiralis* + *T. nativa*, *T. britovi* + *T. nativa*), and *T. pseudospiralis* was never found in mixed infections.

DISCUSSION

From the present study it is clear that in epidemiological studies the multiplex PCR has the advantage over the RAPD-PCR in being faster and easier to interpret due to a simple and distinct band pattern but also that this technique is less subjective and much less influenced by sub-optimal preservation of the parasite material. This difference was most obvious in the detection of mixed infections, but due to the relative few individual larvae tested, the variation might also be explained by chance. Since the two PCR techniques differ mainly with regard to primer composition and are equally laborious, the multiplex PCR can be recommended for epidemiological studies. Thus, even though the correlation between the two tests is high under ideal conditions, the multiplex PCR permits more reliable identification of material that has been frozen and thawed repeatedly and subsequently preserved in ethanol.

For epidemiological studies, sub-optimally preserved samples may be frequent, and the multiplex PCR pro-

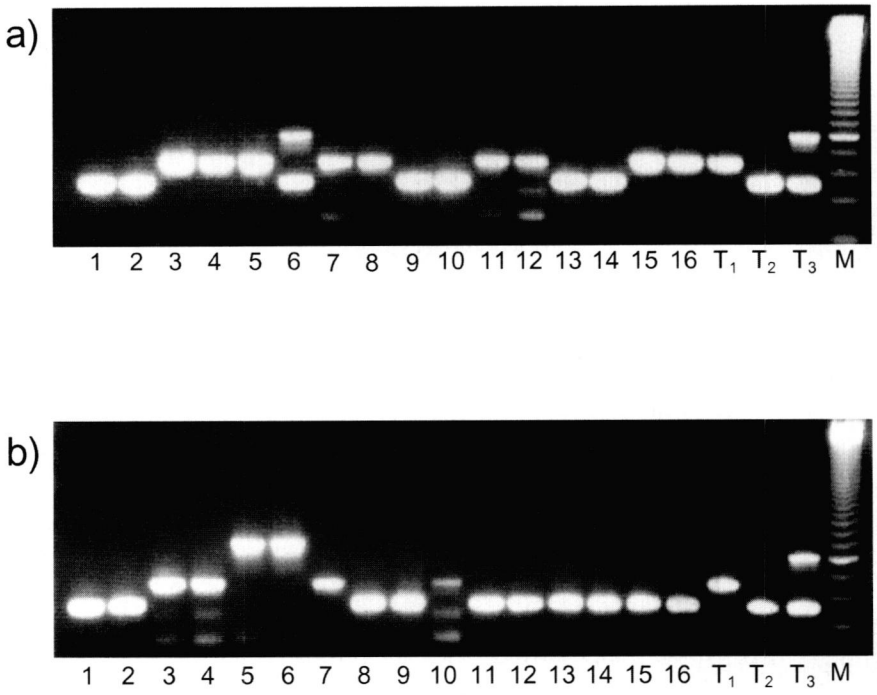

Fig. 2. – a-b: Examples of multiplex PCR patterns obtained with crude preparations of individual *Trichinella* muscle larvae. Reference larvae: T1, *Trichinella spiralis*; T2, *Trichinella nativa*; T3, *Trichinella britovi*. M: 100 bp ladder (Amersham-Pharmacia biotech).
a: Lanes: 1+2 (fox): *T. nativa*; 3+4 (pig), *T. spiralis*; 5+6 (Raccoon dog), *T. spiralis* and *T. britovi*; 7+8 (rat), *T. spiralis*; lanes 9+10 (raccoon dog), *T. nativa*; 11+12 (pig), *T. spiralis*; 13+14 (raccoon dog), *T. nativa*; 15+16 (raccoon dog), *T. spiralis*.
b: Lanes: 1+2 (fox): *T. nativa*; 3+4 (rat): *T. spiralis*; lanes 5+6 (raccoon dog): *T. pseudospiralis*; 7 (pig), *T. spiralis*; 8+9 (raccoon dog): *T. nativa*; 10 (rat), *T. spiralis*; 11+12 (fox), *T. nativa*; 13+14 (fox), *T. nativa*; 15+16 (fox), *T. nativa*.

vides a major advantage for the utilisation of valuable material. For example, due to repeated thawing and freezing, half of the present rats samples (13 of 28) would not have given interpretable results in the RAPD-PCR and left room for speculation. Under routine conditions, when *Trichinella* is found, larvae might not be preserved immediately or simply stored refrigerated in water.

Whereas *T. spiralis* and *T. nativa* have previously been found in Finland, *T. britovi* and *T. pseudospiralis* are new records for Finland. Further, *T. pseudospiralis* is found for the first time in Northern Europe. As in other highly endemic areas, the present study demonstrated that rats, cats, foxes, raccoon dogs, and wild boars may be infected with the domestic species *T. spiralis*, but still we have evidence that it is maintained there and transmitted back to the domestic environment. The absence of sylvatic *Trichinella* in domestic pigs might have several epidemiological explanations, but might also be explained by the very limited infectivity of *T. nativa, T. britovi,* and *T. pseudospiralis* in experimentally infected pigs and wild boars (Kapel & Gamble, 2000; Kapel, 2000). The detection of *T. pseudospiralis* in one sylvatic wild boar, could be the result of host malnourishment, environmental stress or other immuno-suppressing factors in the natural habitat, as suggested for observations of *T. nativa* in other naturally infected wild boars (Pozio & Kapel, 1999).

Not surprisingly, *T. nativa* is the most prevalent of the sylvatic *Trichinella* species, probably because of its high freeze resistance in naturally infected carnivores (Kapel *et al.*, 1999) and the sub-arctic climate of Finland. The absence of *T. nativa* in the rat population is most likely explained by its limited infectivity to rats (Pozio *et al.*, 1992), and the synanthropic nature of this rodent.

Concurrent infections have previously been described with *T. britovi* and *T. nativa* in raccoon dogs and foxes (Pozio *et al.*, 1995, 1998) and with *T. spiralis* and *T. britovi* in wild boars (Pozio *et al.*, 1997), but it is the first time that mixed infections with *T. spiralis* and *T. nativa* have been found. The natural occurrence of such mixed infections may imply that protective immunity from an initial infection may not always persist. The finding that mixed infections occurred most frequently in raccoon dogs is in accordance with its scavenging nature; it was the only host from which all four *Trichinella* species were recovered.

The relatively high prevalence of the non-encapsulating *T. pseudospiralis* is surprising since the species has never previously been recorded from the Northern part of Western Europe. The epidemiology of this species is not clear, but as the only *Trichinella* species able to infect birds, its transmission potential might be underestimated. Its apparent wide distribution in Europe should be considered as a threat to human health in light of the low sensitivity of the classical compressorium technique in the detection of non-encapsulating *Trichinella*. *Trichinella pseudospiralis* in meats of feral pigs have been reported as the source of human trichinellosis (Jongwutiwes *et al.*, 1998; Ranque *et al.*, 2000).

Because we have identified four species of *Trichinella* in Finland, more comprehensive animal prevalence stu-

dies from all Finnish provinces need to be conducted in order to clarify the epidemiology of this zoonosis. Such studies would contribute importantly to the understanding of the transmission of trichinellosis between wildlife and production animals in highly endemic areas sympatric for several *Trichinella* species.

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