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PCR-RFLP patterns of four isolates of *Trichinella* for rDNA ITS1 region

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Abstract: We have studied the genetic differences among four isolates of *Trichinella* including a new strain of *Trichinella spiralis* (ISS 623) recently found from a human case who took a badger in Korea. Because they have a different host origin and came from geographically separated regions, we supposed the genetic pattern of the isolates might be different as had been previously reported. It was analysed by PCR-RFLP analysis of the rDNA repeat that can readily distinguish a species or strain from others. Isolated genomic DNA of each isolate of *Trichinella* larvae was amplified with ITS1 specific primers and digested with restriction endonucleases. The PCR product of ITS1 was confirmed using Southern blot analysis to be a 910 bp fragment. The restriction fragments of each isolate had variable patterns when it was digested with *Rsa* I only. According to the RFLP patterns, the estimated genetic divergence between each isolate was different. In conclusion, four isolates of *Trichinella* including a new strain of *T. spiralis* obtained from a Korean patient may have genetic differences in the ITS1 region and the Shanghai isolate was genetically more similar to the Japanese unknown isolate than others in the ITS1 region.

Key words: *Trichinella*, polymerase chain reaction, random amplified polymorphic DNA technique, ribosomal DNA

INTRODUCTION

Trichinosis continues to be a serious risk for man in some parts of the world. In Korea, human trichinosis outbreak was observed in 1997 at the first (Sohn et al., 2000). The genus *Trichinella* has a broad geographical background and host range, and different isolates can often display distinct biological characteristics. Variability within the genus *Trichinella* has been reported in terms of morphology, infectivity, virulence, antigenicity, and enzyme polymorphism. During the last years, diversity has been analysed using parasitological, biological, morphological, immunological and biochemical (allozyme electrophoretic) parameters (Flockhart et al., 1982; Pozio, 1987; Bolas-Fernandez and Wakelin, 1990).

DNA technology has also provided useful approaches to reveal genetic difference within the genus. Polymerase chain reaction (PCR)-based methods are popular technique recently (Chambers et al., 1986; Soule et al., 1993; Minchella et al., 1994; Zarlenga et al., 1999). It has been reported that PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) analysis of the rDNA repeat can readily distinguish a species or strain from others (Boyd et al., 1989; Zarlenga and Dame, 1992; Gasser and Monti, 1997; Nagano et al.,

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1999). The rDNA internal transcribed spacer (ITS) gene family consists of regions of highly conserved gene sequences that is flanked by transcribed and non-transcribed spacer regions, which may contain variations that can be useful for identifying the relatedness of strains. The ITS1 (internal transcribed spacer 1) PCR product used for a genetic marker to show differences among isolates studied here spans the ITS1 region of the rDNA repeat unit and includes most of the 5.8S gene and a portion of 18S gene (Bowles et al., 1994). Here we have used the PCR-RFLP analysis of ITS1 rDNA and examined the patterns of four isolates of *Trichinella*.

MATERIALS AND METHODS

Parasite

Worms of four isolates of *Trichinella* were maintained by numerous passage in laboratory SD (Sprague-Dawley) rats.; The Shanghai isolate from a domestic pig (presumed *T. spiralis*), the Thai isolate from a wild boar (*T. spiralis*, ISS 411), the Japanese unknown isolate from a raccoon dog (presumed *T. spiralis*) and Korean isolate recently found from a human who took a badger in Korea (*T. spiralis*, ISS 623). The *Trichinella* infected rats were sacrificed and digested in 1% pepsin-HCl at 37°C. After through several washing in distilled water, muscle larvae were collected under microscope avoiding contamination of host tissue debris and frozen at -70°C until DNA isolation.

Isolation of genomic DNA

Genomic DNA from each isolate was purified as described by Rodriguez et al. (1996) with minor modifications. Previously, the parasites were homogenized under liquid nitrogen with lysis buffer (50 mM Tris-HCl buffer pH 8.0, 50 mM ethylene diamine tetra-acetic acid (EDTA), 100 mM NaCl, containing 0.5% sodium dodecyl sulfate (SDS)). Then the homogenate was incubated with 100 μg/ml proteinase K (Sigma, USA) for 3 hours at 37°C. After an equal volume of phenol-chloroform extraction, the aqueous phase was digested with 150 μg/ml RNase (Sigma) for 30 minutes at 37°C. Additional phenol-chloroform extraction and chloroform extraction was performed subsequently. Total genomic DNA was precipitated by ethanol extraction and the DNA was resuspended in sterilized water. DNA concentration was determined by standard procedures.

PCR amplification and Southern blotting

A standard PCR reaction was performed using forward (5′ GTCGTAACAAGGTTTCCGTA 3′) and reverse (5′ TCTAGATGCGTTCGAA(G/A)TGTCGATG 3′) oligonucleotide. The internal transcribed spacer 1 (ITS1) specific primers were designed (Bowles and McManus, 1993) by comparing conserved rRNA gene sequences from a range of organisms. Genomic DNA was amplified in a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM of each dNTP and 2.5 units Taq polymerase (TaKaRa, Shiga, Japan). The following cycling conditions were used: 95°C, 30 sec (denaturation); 58°C, 1 min (annealing); 72°C, 1 min (extension) for 35 cycles on a thermocycler (MJ research). The PCR products were checked on agarose gel with ethidium bromide staining and transferred onto nylon membrane by standard capillary blotting method with 10 x SSC. Then the blotted membrane was hybridized with 5.8s rDNA probe made by PCR amplification with specific primers (forward: 5′-GGTACCGGTGGATCACT CGGCTCG-3′, reverse: 5′-TCTAGATGCGTTC GAA(G/A)TGTCGATG-3′). It was detected by ECL™ detection system (Amersham, Buckinghamshire, England) as described in manual.

Restriction endonuclease digestion

The 4-base recognizing restriction endonuclease for digestion of the ITS1 fragment were used.: *Alu* I, *Cfo* I, *Msp* I, *Rsa* I and *Taq* I. The PCR products were digested with several restriction endonucleases and analysed on agarose gel with ethidium bromide staining.

Analysis of ITS1 PCR-RFLP

Individual gel images of RFLP bands were analysed using phylogeny analysis program, RESTSITE v.1.2 (Miller, 1994). The estimated

genetic divergence was calculated using CALCD and the dendrogram was constructed by UPGMA v.2.0 (Miller, 1994).

RESULT

The PCR products of the ITS1 region of all *Trichinella* isolates consisted of a fragment of approximately 910 bp and an additional band (525 bp) in the Japanese unknown isolate (Fig. 1A). It was confirmed that the anticipated ITS1 product is indeed a 910 bp fragment by Southern blotting analysis with 5.8s rDNA probe that is included in ITS1 sequences (Fig. 1B). The PCR products were digested with *Alu* I, *Cfo* I, *Msp* I, *Rsa* I, and *Taq* I. The RFLP pattern of four isolates digested using *Alu* I and *Msp* I were all the same at 910 bp uncut while digestion with *Cfo* I and *Taq* I produced the same two bands, 653 bp and 210 bp in every isolate respectively (Fig. 2). But the pattern digested with one of 4 base recognizing enzymes, *Rsa I*, distinctly showed the different patterns in ITS1 of four isolates. The Shanghai isolate showed a 308 bp band, the Thai isolate had two bands at 392 bp and 336 bp, the Japanese unknown isolate had 372 bp and 308 bp band, while the Korean isolate had bands of 385 bp and 313 bp (Fig. 3).

Estimated genetic convergence was analysed using RESTSITE v.1.2 (Table 1). Genetic divergence between the Korean isolate and Shanghai isolate was 0.027218. It was revealed genetic divergence between the Korean isolate and the Japanese unknown isolate and between the Korean isolate and the Thai isolate was 0.03488 and between the Shanghai isolate and the Japanese unknown isolate was 0.008005. The phyllogenic dendrogram was drawn using UPGMA v.2.0 as shown in Fig. 4.

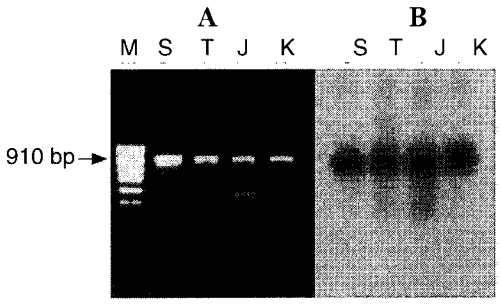

Fig. 1. PCR products of the ITS1 region of four isolates of *Trichinella* (A). Southern blot analysis probed by 5.8S rDNA (B). M, 100 bp ladder; S, Shanghai isolate; T, Thai isolate; J, Japanese unknown isolate: K. Korean isolate

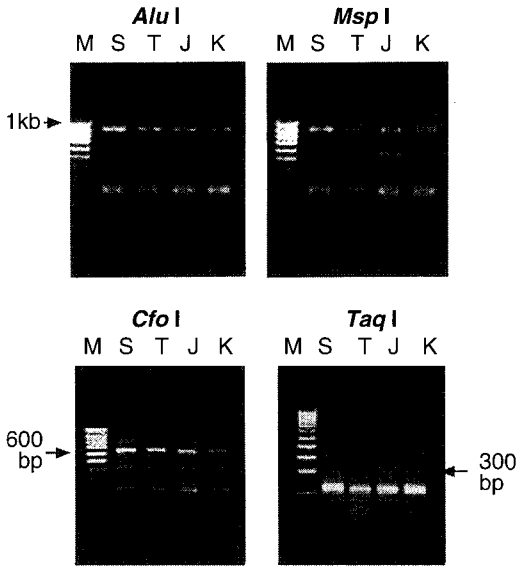

Fig. 2. PCR-RFLP patterns of ITS1 region digested with *Alu* I, *Msp* I (uncut; upper pannel), *Cfo* I and *Taq* I (lower pannel). It shows a similar patterns among four isolates of *Trichinella*. M, 100 bp ladder; S, Shanghai isolate; T, Thai isolate; J, Japanese unknown isolate; K, Korean isolate

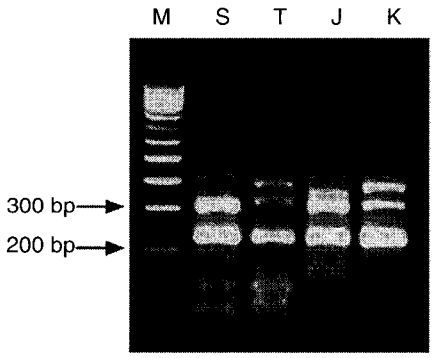

Fig. 3. PCR-RFLP of four isolates digested with *Rsa* I. M, 100 bp ladder; S, Shanghai isolate; T, Thai isolate; J, Japanese unknown isolate; K, Korean isolate

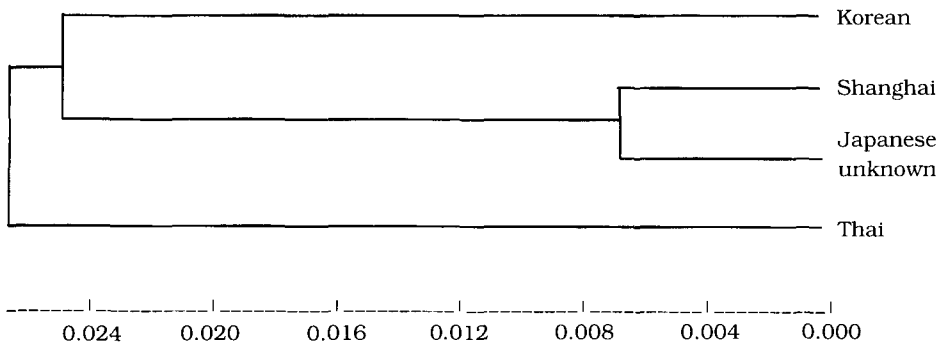

Fig. 4. Dendrogram of estimated genetic divergence. UPGMA v.2.0

Table 1. Estimated genetic convergence among four isolates of *Trichinella* using CALCD, RESTSITE v 1.2.

	Korean	Shanghai	Japanese unknown	Thai
Korean				
Shanghai	0.022328			
Japanese unknown	0.028797	0.006714		
Thai	0.028797	0.022328	0.028797	

DISCUSSION

In this study, we have shown the genetic difference of four isolates of *Trichinella* using PCR-RFLP analysis. The genus *Trichinella* has been tried to classify to species. Thus, nameless taxa (T5, T6 and T8) have been compared with already known species using various molecular and biochemical methods including RFLP (Wu et al., 1999), RAPD (random amplified polymorphic DNA; Bandi et al., 1993), SSCP (single-stranded conformational polymorphism; Gasser and Monti, 1997) and isoenzyme assay, etc. (La Rosa et al., 1982). These methods were also applied to the classification of many subspecies and isolates obtained from geographically distinct areas or different hosts. Dame et al. (1987) grouped 18 isolates of the *T. spiralis* from various origin to subspecies, *T. spiralis spiralis* and *T. spiralis* spp. by probe hybridization to restriction fragments of genomic DNA. If *T. spiralis* isolates in the present study were compared with appropriate reference subspecies, they might have also been separated into the subspecies groups as known.

It is not surprising as heterogeneity among repeat units of rDNA is characteristic of eukaryotic DNA. Bowles et al. (1994) genetically characterized several strains of *Echinococcus granulosus* through PCR-RFLP analysis of the mitochondrial DNA as well as the nuclear ITS1 region and reported that the ITS1 region of rDNA repeat can distinguish the cervid strain from other strains of *E. granulosus*. For the species *T. spiralis*, it is apparent that the internal repeats of the IGS (intergenic spacer) and ITS region vary between isolates and are thus useful in distinguishing from others (Boyd et al., 1989).

Here we tried to characterize the molecular genetic differences on the nuclear ITS1 region between the newly found Korean isolate and other isolates maintained experimentally. The results showed that the ITS1 PCR product amplified with ITS1 specific primers was identical in four isolates and Japanese unknown isolate had an additional band. Southern blot analysis confirmed that the band in all four isolates is the appropriate ITS1 product. And it's restriction enzyme fragments were different from each other when

only one of the restriction enzymes, *Rsa* I, was reacted. It would appear that small genetic changes had occurred during the courses of evolution. However, it is not known at the present whether these differences are of any significance.

The estimated genetic divergence between the isolate obtained from a Korean patient and the Thai or the Japanese unknown isolate was comparatively higher than between the others. The result indicates that the Shanghai and Japanese unknown isolates are significantly genetically closer due to their proximity on the phyllogenic dendrogram. Thus the Shanghai isolate may be genetically more closer to the Japanese unknown isolate than others. It was thought that geographic location may contribute to the diversity in development of each isolate. Dame et al. (1987) had determined that genetic differences do exist between sylvatic isolates and domestic isolates. In this vain, it is important to note that the origin of the Shanghai isolate is domestic (from the domestic pig) while the other isolates are known to be from wild hosts (the Korean strain originating from ingestion of a wild badger). However, there appears to be no significant relations with differing origins in our study. According to the dendrogram analysed, the Thai isolate may have evolved earlier than the others. This may be due to the geographic isolation of that isolate. In addition, it is suggested that Korean isolate is genetically distinct from the other three isolates presumed *T. spiralis* (Shanghai, Thai and Japanese unknown isolate) from foreign countries and that three isolates also differ from one another.

In conclusion, it can be stated that the four isolates of the *Trichinella* genus display genetic difference in their respective ITS1 regions of rDNA. However the significance of these observations can only be determined when the rDNA of the various *Trichinella* isolates is sequenced. In turn, it will be possible to determine if the genetic differences found during RFLP analysis are due to differences in genetic development in the whole range of the gene or just due to point mutations. Further PCR-based molecular studies of other genes and sequences must be performed for proper molecular characterization and identification of subspecies of *T. spiralis*.

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