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Occurrence of Variants of Repeated DNA Sequences in *Trichinella spiralis*

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Abstract

We amplified a 602 base pair (bp) fragment (reportedly a repeated sequence) of genomic DNA of *Trichinella spiralis* by means of the polymerase chain reaction (PCR). Gel electrophoresis of the amplified fragments yielded the expected band (DNA fragment) of 602 bp plus three bands, ca 800, ca 440, ca 230 bps. Sequence analysis of the ca 230 bp fragment revealed that it has 247 bp composed of two lesser portions, separated by a third but missing fragment, residing in the 602-bp fragment. The 602-bp fragment apparently was the site where the deletion occurred due to "slipped mispairing" of nucleotides during mitosis/meiosis, because both the 5' and 3' ends of the missing DNA fragments shared as many as 14 consecutive base pairs. Detection of the new sequences raises the possibility of establishing new criteria for the taxonomic classification of *T. spiralis*.

Key words: *Trichinella spiralis*, taxonomy, mutation, DNA, repetitive sequence, PCR

Introduction

Recent progress in genetic engineering technology, namely PCR, enabled the detection of even single copies of genes in a minute samples of DNA. This powerful tool has been applied to the diagnosis of diseases including parasitic infection such as toxoplasmosis (Holliman *et al.*, 1990) and trypanosomiasis (Avila *et al.*, 1991). PCR might also be useful in detecting *Trichinella spiralis*, which infects most all vertebrates, including humans, Trichinosis remains a severe public health problem throughout the world. Kassen *et al.* (1986) reported a 1.7 kb repetitive DNA sequence family was strain-specific in *T. spiralis*. Repetitive sequence is probably the best candidate template DNA for a diagnostic PCR test because its abundance minimize false negative results. For example, the 1.7 kb repetitive sequence occurs in minimally dispersed direct tandem arrays and has a copy number as high as 2,800, constituting 2% of the 2.5×10^8 bp haploid genome (Klassen *et al.*, 1986). A major portion (1.6 kb) of 1.7 kb fragment has been sequenced by the same group (deVes *et al.*, 1988), and the PCR method successfully augmented the 602 bp fragment contained within that sequence. Klassen *et al.* (1986) concluded that this 1.7 kb fragment was useful in identification of the *Trichinella* strain. But their PCR experiments also produced two unexpected fragments of ca 800 and ca 230 bps, which they regarded as variant forms of the fragments of 620 bp. In this study we successfully sequenced one of the unexpected fragments, and comparison with the sequence obtained by deVos *et al.* (1986) led to the conclusion that it originated from the fragment of 602 bp during the events of mitosis/meiosis. The possibility is discussed that this simple and straightforward process not only identifies infectious agents but also may shed lights on the course of strain evolution in *Trichinella*.

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Materials and Methods

A Polish strain (an isolate generated in Poland) of *T. spiralis* was maintained in ICR mice and isolated by pepsin-HCl digestion. Excysted larvae were suspended in a Tris-HCl buffer (pH 7.4) containing 0.5% N-lauroylsarcosine and 20 mM EDTA, frozen

in a deep-freezer (–80°C) and mechanically homogenized. Genomic DNA was isolated by conventional methods, which included proteinase K incubation at 50°C for 2 hours followed by phenol treatment and ethanol precipitation.

Two oligonucleotides were synthesized with a MODEL 391 PCR-MATE DNA SYNTHESIZER® (Applied Biosystems, Tokyo) according to the manufacturer's protocol. Both sequences, 5'-TCGAATTCGTAAAGCGGTGGTGCGTATTCCAT (first TCGAATTC is EcoRI site for ligation purposes) and 5'-AGTTTGCATACCGAACAACCGCTC are highlighted in Fig. 2. Using these as primers, PCR amplification was carried out in 30 cycles using a GeneAmp® PCR reagent kit with AmpliTaq® DNA polymerase (UNITED STATES BIOCHEMICAL CORPORATION, Cleveland OH). Each cycle consisted of denaturation at 94°C for 2 min, followed by annealing at 46°C for 1 min and extension at 77°C for 2 min.

Amplified DNA fragments were electrophoresed in 4% NuSieve 3:1® (FMC BioProducts Rockland, ME) supplemented with ethidium bromide. For sequence analysis, another sample of the amplified DNA was then electrophoresed in a low temperature melting gel and the DNA fragment in each band was isolated. After purification by ethanol precipitation and digestion with EcoRI, the DNA fragments were ligated to pGEM vectors that had been previously digested with EcoRI and Hinc II. This step was followed by transformation of competent *E. coli* (MC 1061), and recombinant DNA was isolated. After alkaline denaturing the single-stranded DNA fragments were sequenced with an A.L.F. DNA sequencer® (Pharmacia Biosystem, Uppsala, Sweden).

Results

Electrophoresis of the PCR products produced at least four bands of ca 800, 602, ca 440 and 247 bps (lane A in Fig. 1). For a marker of bps, ø × 174/Hae III digest (Toyobo Co., Ltd., Osaka Japan) was run in parallel (lane B in Fig. 1). The relative intensity of each band was determined by means of Densitograph® (Atto Co., Ltd. Tokyo Japan) as shown in the upper panel of Fig. 1. The result of sequence analysis of the 247-bp fragments is given in the upper line of Fig. 2. The sequence was compared with that of the 602-bp fragment reported by deVos *et al.* (1986) (in the lower line of Fig. 2).

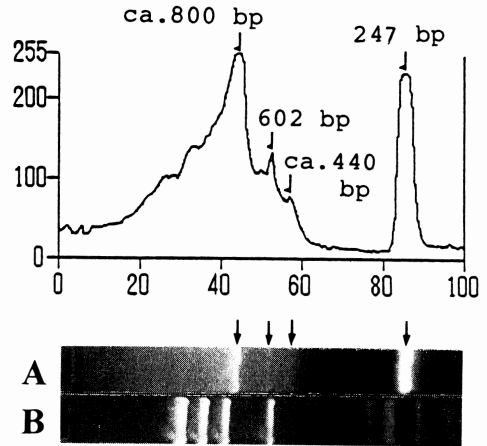

Fig. 1 The upper panel shows relative intensity (vertical axis) of bands after electrophoresis (horizontal axis) of the PCR products. The lane A is band pattern of PCR products. The lane B is a reference of the number of base pairs including 1353, 1078, 872, 603, 310, 281, 234 and 194 bps. Arrows indicate positions of each band.

Knowing the sequence of the 247-bp fragment, we searched for it in the 1.6 kb sequence of the parent DNA reported by deVos *et al.* (1986). The fragment was located in the 602-bp fragment between the two primers, but it was split into two parts, with one part comprising nucleotides 1–217 and the other part comprising nucleotides 573–602 of the fragment of 602 bp. The sequence of the fragment of 247 bp isolated in the present study was identical to that residing in the 1.6 kb parent DNA reported by deVos *et al.* (1986). The intervening DNA fragment made up of nucleotides 218–572 (shown as bars in Fig. 2) was not within the 247-bp fragment we isolated.

Discussion

The primers reported by Dupouny-Camet *et al.* (1991) seem to be the only ones currently available tha can detect repetitive DNA sequences in *Trichinella*, and are designed to amplify the fragment of 602 bp using genomic DNA of *Trichinella spiralis spiralis*. However, in the study by Dupouny-

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present sequencing    GTAAAGCGGTGGTGCGTATTCCATTCTTGAGC
deVos et al. (1986)   GTAAAGCGGTGGTGCGTATTCCATTCTTGAGC
                      ↑1

CACACCATTAATGTGATTGGCTATTTATTTTGCAACCTATTTTATCGAATTTCATCTCTC
CACACCATTAATGTGATTGGCTATTTATTTTGCAACCTATTTTATCGAATTTCATCTCTC

AATTTTACACATTCATACCTATGCTTTTCCACCGGGAATGGTATTAATCGCTTCACCGCC
AATTTTACACATTCATACCTATGCTTTTCCACCGGGAATGGTATTAATCGCTTCACCGCC

ATGTCTGTAACACCAGTAAATACTCGTGAAGCTTTGCTAATCATTGCATTTCACAGCGCC
ATGTCTGTAACACCAGTAAATACTCGTGAAGCTTTGCTAATCATTGCATTTCACAGCGCC

CTAAC-------------------------------------------------------
CTAAC[GAATATGAGCGGTT]TGTCGATGTGTAAACTATAATAAGGGTTCTTCTTACCAATT
     ↑217          ↑231                          ↑259

------------------------------------------------------------
TTCCGGATTTGTACCTGTGTTTCTCCGCACACATGTTTGGTAATGGTTCCTCTCTATGGA

------------------------------------------------------------
ATGTAATACTCTCTAATATAGTCGAGCATTGTTAATCATTCCATTTCAGAGCGCTCCAAG
                     ↑354                ↑374    ↑382  ↑388

------------------------------------------------------------
GAATATACACGGTCAATCGGTACACAAACTCTGGTAAGCAGTTACTCTTACCAATATTGC
     ↑398                                  ↑438

------------------------------------------------------------
GGATTTCTACCAGAGTTTCTCCCCACAGATGTCAGGTAATGTTGCCTCTCTATGGCCTGC

------------------------------------------------------------
TATACTTTCTAAGATAGGTAAACATGTTAATCTTTGCATTTCATTTCAGACAGCACCAAG
                 ↑529                     ↑554    ↑562  ↑568

GAATATGAGCGGTTGTTCGGTATGCAAACT
[GAATATGAGCGGTT]GTTCGGTATGCAAACT
↑573  ↑579    ↑586            ↑602
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Fig. 2 Nucleotide sequence of the 247 and 602 bp is shown in the upper and lower lines, respectively. Missing nucleotides in the 247-bp fragments are indicated by bars. The boxed fragments occur twice in the 602-bp fragment. Highlighted nucleotides are primers used.

Camet *et al.* (1991), the primers produced two unexpected fragments of ca 800 and ca 230 bps as well as the expected fragment. Applying the same primers to the genomic DNA of a Polish strain of *Trichinella spiralis*, we obtained nearly identical results except for an additional unexpected fragment of ca 440 bp. It is a matter for future investigation to determine whether or not their 230-bp fragment is analogous to our 247-bp fragment.

Production of the expected fragment of 602 bp further suggests that the primer originated by Dupouny-Camet *et al.* (1991) may be useful in detecting the infectious agent in question as well as being useful in genomic DNA cross-hybridization tests (Garate *et al.*, 1991). Before the PCR procedure can be fully applied, however, the mode of occurrence of repetitive DNA in whole genomic DNA should be elucidated. Such information may shed light on the mechanism by which other unexpected bands appear in PCR amplification.

The occurrence of the shorter DNA fragments in our study can be accounted for by the deletion of a fragment from the parent DNA sequence. Two identical fragments of 14 nucleotides with the sequence GAATATGAGCGGTT (boxed fragments in Fig. 2) were found at both the 3' and 5' ends of the missing portion of the 1.6 kb DNA sequence. Such occurrence of the identical nucleotide sequences often results in accidental mispairing of bases during DNA replication (Nalbantoglu *et al.*, 1986). Because direct repeat sequences at each terminus tend to form a cross-over point to produce a novel junction leaving only one copy of the repeat. In fact only one copy of the repeat has been retained in our fragment of 247 bp. Alternatively, the deletion may be induced by the recombinational events in mutation (as reviewed by Meuth, 1990).

Since a repetitive sequence is not functional, the resulting mutant organisms may not be lethal, in which cases mutated DNA sequence will likely be retained in the population and passed on by offspring to subsequent generations. Therefore these repetitive sequences can be used to trace the family tree of the *Trichinella* strain in question, or can even serve as additional criteria for identifying the strain. For example, the presence of the fragment of ca 440 bp among the PCR products of our polish strain of *T. spiralis* suggested that it is different from *T. spiralis* strain that Dupouy-Camet *et al.* (1991) used.

Genetic differences among the various isolates of *Trichinella*, which would warrant classifying them as distinct species, have long been sought to end the controversy over whether "new" forms are varieties, strains, sibling species, subspecies, or species persisted. Traditionally, the criteria for classifying isolates (or strains) of *T. spiralis* have been based on the differences in virulence, interbreeding ability, isoenzyme profile (Mydynsky and Dick, 1985), and resistance to high temperature (as reviewed by Dick, 1983). But the lack of clear-cut morphological differences made it difficult to distinguish between *Trichinella* isolates from various sources by inspection of one individual. If the unexpected bands of PCR-amplified DNA are results of deletion, the PCR of repetitive sequence may be a promising probe. The occurrence of the deletion, however, should not be overemphasized because the deletion itself is not enough to justify new classification. The taxonomic significance of the banding patterns could possibly be established after correlations are made with the referring strains.

The 602 bp sequence is interesting in that it contains many sites where deletion is likely to occur. Because the sequence of CCAAGGAATAT occurs twice, corresponding to nucleotides 388–398 and 568–579, and CATTTCAGA also occurs twice, at nucleotides 374–382 and 554–562. Sequences 259–354 and 438–529 also showed a high degree of homology (82%). If repeated sequences are targets of deletion as suggested by Nalbantoglu *et al.* (1986), they offer more probes for studying the taxonomy of *T. spiralis* strain. Our laboratories are currently engaged in sequencing the rest of the unexpected bands.

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