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Further Justification of Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) for Use of Genomic Analysis of *Trichinella*

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Abstract

An arbitrarily primed polymerase chain reaction (AP-PCR) revealed DNA polymorphism (random-amplified polymorphic DNA, RAPD) among eight strains of *Trichinella* which included Polish, Yamagata, Polar, USA, Thai, Iwasaki, *T. pseudospiralis* and Changchun strains. Such DNA polymorphism well correlated to phenotype difference including antigenicity, isozyme pattern, and cyst-forming ability. Therefore, AP-PCR seemed to be a rational and reliable means to afford additional criteria for taxonomy of *Trichinella* species.

Key words: *Trichinella*, AP-PCR, RAPD, taxonomy

Introduction

Every biological being is genetically controlled by its genomic DNA. The same is true for parasites. Genotypic alterations are likely associated with phenotypic variations. Thus DNA analysis is theoretically a most reliable means as a criteria of taxonomy of parasites. Reportedly, DNA analysis methods adapted in taxonomical study include pulse field electrophoresis of genomic DNA after endonuclease digestion, and restriction fragment length polymorphism (RFLP) and random-amplified polymorphic DNA (RAPD) by means of an AP-PCR.

AP-PCR is a simple and straightforward technique to analyze genome without knowing any sequence of sample DNA (Welsh *et al.*, 1990; Williams *et al.*, 1990). The trick of this technique is PCR with an arbitrarily determined primer (10 base pair oligonucleotide). The annealing temperature is low compared to that of PCR with regular sized primer (about 20 bp). Although the primer is arbitrarily determined, substitution of even one nucleotide at the 3' end of the primer results in an obvious difference in the banding pattern of PCR products, suggesting AP-PCR is one of the most sensitive ways to detect DNA polymorphisms between and within isolates of interest. Consequently, RAPD revealed by AP-PCR has been used as a fingerprint of parasites that cannot be typed by conventional criteria.

Taxonomical classification, of course, should be based on phenotypic characteristics including morphology, life cycle, virulence, antigenicity, and isozyme pattern. Therefore, genomic criteria can be justified only when it is linked to such visible difference. In this contribution we show DNA polymorphism revealed by AP-PCR fulfills such requirements. The sample used was 8 strains of *Trichinella* whose phenotype was well defined by previous papers (Fukumoto *et al.*, 1987; Fukumoto *et al.*, 1988; Saito and Sendo, 1989; Saito *et al.*, 1994).

Materials and Methods

Parasites

Trichinella isolates used in this study includes Polish, Yamagata, Polar, USA, Thai, Iwasaki, *T.*

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pseudospiralis and Changchun strains. The Polish strain was isolated from a wild pig. The Yamagata strain was isolated from a raccoon dog in Yamagata city (Saito *et al.*, 1986). The polar strain was isolated from a bear. The USA strain was isolated from a pig. The Thai strain was isolated from a human. The Iwasaki strain was isolated from a Japanese black bear in Iwasaki village (Yamaguchi, 1975; Yamaguchi, 1978; Yamaguchi, 1991; Yamaguchi *et al.*, 1982). *T. pseudospiralis* was isolated from a raccoon in the Northern Caucasus region. The Changchun strain was isolated from a dog in China.

Genomic DNA preparation

The parasites (about 10,000 larvae) was collected from muscles by extensive digestion with pepsin-HCl. To extract genomic DNA, the muscle larvae were frozen at –30°C, homogenized mechanically by means of hammering at –30°C and suspended in SE buffer (0.5% Sarcosyl, Sodium N-Lauroyl sarcosine plus 20 mM EDTA in 20 mM Tris-HCl, pH 7.4), followed by digestion with 50 μg/ml Protease K (Merck, Darmstadt, Germany) in SE buffer at 37°C for more than 3 hours. The solution was mixed with the same volume of phenol (TE Saturated Phenol purchased from Wako Pure Chemical Industries, Osaka, Japan) for 10 min at room temperature and centrifuged at 3,000 rpm for 15 min with a Tomy High Speed Microcentrifuge MR-150 and a TMA-2 rotor (Tomy Seiko, Tokyo, Japan). The supernatant was recovered and mixed with the same volume of chloroform (at 24:1 mixture of chloroform and isoamylalcohol) for 10 min at room temperature. The solution was centrifuged at 3,000 rpm for 15 min with the same centrifuge, and the resulting supernatant was subjected to ethanol precipitation at –30°C for more than 2 hours. This was centrifuged at 14,000 rpm for 15 min with the same centrifuge. The resulting genomic DNA was suspended in TE buffer (1 mM EDTA in 10 mM Tris-HCl, pH 7.4) to give the final concentration of 2 ng/μl, and frozen until use.

PCR condition

PCR was performed using the genomic DNA as a template and 10 base pair oligonucleotides as an arbitrary primer including GC100 (5'-CGCCCCC-GGC-3'), GC90 (5'-CGGCCCGGT-3'), GC80 (5'-CGGCCCCTGT-3'), GC70 (5'-CGGCCACTGT-3'), GC60 (5'-CGGTCACTGT-3'), GC50A (5'-TGGTCACTGT-3'), GC50B (5'-GCAAGTAGCT-3'), and GC50D (5'-ACGTTCAGTG-3'). PCR reagents were purchased from TaKaRa Shuzo Co. Ltd. (Kyoto, Japan). The reaction mixture was composed of 16 μl of dNTPs (2.5 μM), 5 μl of 10x PCR buffer, 0.2 μl of *Taq* DNA polymerase, 5 μl of the template DNA (2 μg/ml), 5 μl of primers (5 μM) and 30.8 μl of distilled water. This was overlaid with a drop of mineral oil (Sigma Chemical Co., St. Louis, MO, USA). A thermal reactor (Zymoreactor II purchased from ATTO Corporation, Tokyo, Japan) was programmed for 45 cycles of denaturation at 92°C for 30 sec, annealing at 36°C for 60 sec and extension at 72°C for 60 sec. The PCR products were electrophoresed in 1.5% agar gel (SEAKEM GTG purchased from FMC BioProducts, Rockland, ME, USA) and detected by staining with ethidium bromide. The molecular size marker, pGEM Markers (Promega Co., Madison, WI, USA), was electrophoresed in parallel to estimate the size of DNA fragments.

Results and Discussion

As shown in Panels A to H in Figure 1, each primer produced complex banding patterns that differ in number, intensity and molecular size of the bands. The characteristic profile is supposed to reflect polymorphism of genomic DNA of each sample, hereby called RAPD by Williams *et al.* (1990). This is supposed to depict similarities and differences between various strains (DNA fingerprint) and applied to identify *Trichinella* species (Bandi *et al.*, 1993a; 1993b; Dupouny-Camet *et al.*, 1994).

The most notable findings were the distinct banding pattern of *T. pseudospiralis* as shown in Fig. 1. The banding pattern was shared by no other samples although some individual bands were common (* in Panel H). Similar results have been reported by Dupouny *et al.* (1994). The peculiar status of *T. pseudospiralis* in phenotype includes isozyme and antigenicity pattern, and the fact that only *T. pseudospiralis* does not form cysts but the other *Trichinella* strains do. Our results seemed to reconfirm such an obvious phenotype difference corre-

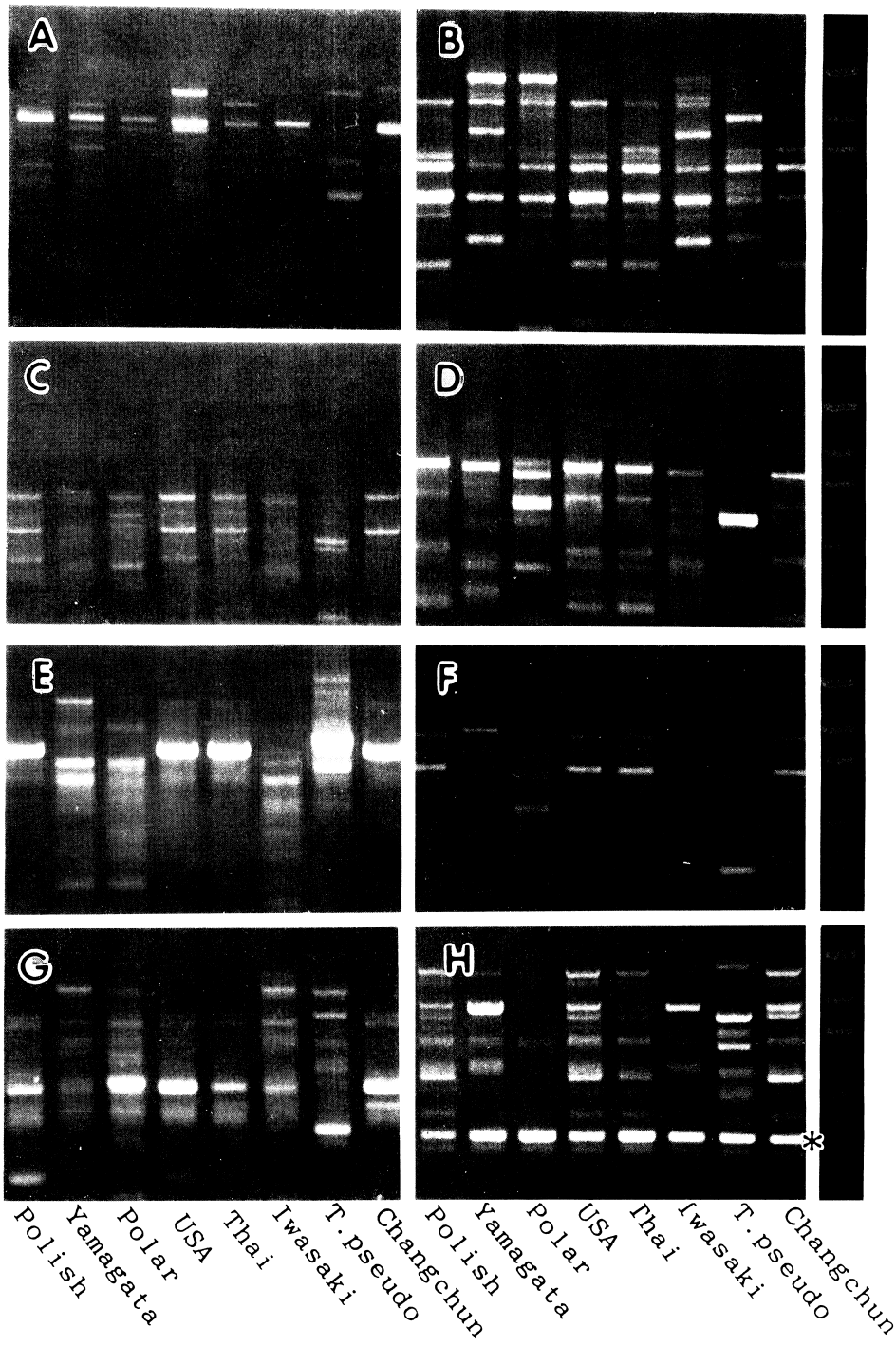

Fig. 1 Random-amplified polymorphic DNA banding patterns of *Trichinella* species with primers GC100, GC90, GC80, GC70, GC60, GC50A, GC50B and GC50D (Panel A to H, respectively). In each panel 8 samples of Polish, Yamagata, Polar, USA, Thai, Iwasaki, *T. pseudospiralis* and Changchun strains are arranged from left to right. Major bands in the molecular size marker include 2,645bp, 1,605bp, 1,198bp, 676bp, 517bp, 469bp.

lated to genotype revealed by AP-PCR.

The rest of the *Trichinella* strains share phenotype more or less. Reportedly strains of Polish, USA and Thai are falled in the same type on the basis of isozyme patterns (Fukumoto *et al.*, 1987; 1988). This resemblance of phenotype characteristic was in agreement with RAPD results with primers GC90, GC80, GC70, GC60 and GC50A (Panel B to F, respectively), but not with primer GC100 (Panel A). The Changchun strain is also falled in the same type based on antigenicity (Saito *et al.*, 1994). This phenotype characteristic was in agreement with RAPD results with primers GC80, GC70, GC60 and GC50A (Panel C to F, respectively).

Strains of Yamagata and Iwasaki were isolated in Japan (Saito *et al.*, 1986; Yamaguchi *et al.*, 1982), and share isozyme pattern (Fukumoto *et al.*, 1987; 1988) and major antigenicity (Saito *et al.*, 1994). The two strains are reproductively isolated from the other strains (Saito and Sendo, 1988). This resemblance of phenotype characteristic was in agreement with RAPD results with primers GC100, GC90, GC80, GC50A and GC50D (Panel A, B, C, F and H, respectively), but not with primer GC60 (Panel E). Although it has been difficult to distinguish Yamagata and Iwasaki strains by conventional criteria, the two can be typable by RAPD pattern.

The Polar strain has some resemblance, but not exact, to both Yamagata and Iwasaki strains in the isozyme pattern (Fukumoto *et al.*, 1987; 1988). This phenotype characteristic was in agreement with the RAPD results with primers GC100 and GC80 (Panel A and C, respectively), but not with primers GC90, GC60, GC50A, GC50B or GC50D (Panel B, E, F, G and H, respectively).

There have been no agreements about which kinds of DNA fragments are amplified in AP-PCR, and even technical ambiguity remains still unsolved (Muralidharan *et al.*, 1993). Also AP-PCR is prone to overemphasize even minor differences that cannot be detected by conventional methods. Thus biological importance of AP-PCR is still a matter of future investigations. Nevertheless the genotype revealed by AP-PCR method well correlated to phenotype difference as shown in this contribution. Therefore DNA polymorphism revealed by AP-PCR is biologically relevant and possibly reflects some difference in phenotypes including antigenicity, isozyme pattern, and cyst forming ability among some isolates of *Trichinella*. In conclusion, our result seems to further convince the use of AP-PCR for taxonomy of *Trichinella* as well as other parasites.

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