USE OF POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF TOXOCARIASIS: AN EXPERIMENTAL STUDY

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Abstract. In this paper we report the usefulness of polymerase chain reaction technique in the diagnosis of visceral larva migrans in a mouse model. Liver samples obtained from two set of experimentally infected mice (10, 100, 1,000 and 10,000 embryonated *Toxocara canis* eggs per mouse) along with the eggs of *T. canis*, *T. cati* and *Ascaris suum* were included in this study. Polymerase chain reaction (PCR) was performed using *Toxocara* primers (SB12). The first PCR product electrophoresis revealed very thin positive bands or no bands in liver samples. However, on second PCR a clear-cut bands were observed. No positive band was shown by *A. suum* eggs. Our findings thus indicate the usefulness of PCR technic in the diagnosis of visceral larva migrans (VLM) in liver biopsy materials specifically by means of double PCR using the primer SB12.

INTRODUCTION

Visceral larva migrans (VLM) resulted due to the *Toxocara* infection accounts for one of the important public health problem both in developed (Uga, 1993) and developing countries (Thompson *et al*, 1986; Rai *et al*, 1996). Diagnosis of VLM presently, is made by detecting *Toxocara* antibodies in serum using either soluble embryonated egg antigen or larval excretory-secretory antigen. *Toxocara* seroprevalence has been reported to be as high as 86.0% elsewhere in the world (Thompson *et al*, 1986). Previously, we also reported a seroprevalence rate of 81.0% in apparently healthy Nepalese (Rai *et al*, 1996). Such a high seroprevalence makes difficult to diagnose an active case of toxocariasis serologically as the antibodies persist for longer period even after the elimination of parasites. Furthermore, immunological cross-reaction with *Ascaris lumbricoides* has also been pointed out by some investigators (Glickman *et al*, 1985; Lynch *et al*, 1988). Thus, it appears to be necessary to use other technics that could be useful in the diagnosis of active cases of VLM specifically. Therefore, with the aim to overcome the present diagnostic problem of VLM, we studied the usefulness of polymerase chain reaction (PCR) technic in the diagnosis of an active case of VLM in experimentally infected mice using the primers developed at our laboratory recently.

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MATERIALS AND METHODS

Preparation of infective *Toxocara* eggs

Toxocara eggs were recovered from female *T. canis* and *T. cati* worms obtained from Hyogo Prefectural Animal Administration Office, Hyogo, Japan and were incubated at 26°C for three weeks for the embryonation.

Infection in mice and recovery of liver

Four female ICR mice aged 8-10 weeks were infected with 10; 100; 1,000 and 10,000 embryonated *T. canis* eggs (in 200 µl of distilled water) orally and liver were recovered after 48 hours post infection. The liver were then frozen at -20°C till processed.

DNA extraction

The parasite DNA was extracted as described previously (Takahashi *et al*, 1995). Briefly, the

liver samples placed into a small plastic bag and frozen at -30°C were crushed mechanically by means of hammering using pre-cooled (at -30°C) iron plate and hammer. The crushed liver tissue was then suspended in SE buffer (0.5% sarcosyl lauryl sarcosamine and 20 mM EDTA in 20 mM Tris-HCl, pH 7.4). An aliquote of homogenized tissue transferred into a micro centrifuge tube (Eppendorf, Germany) was subjected to sodium hydroxide (0.1 M) digestion. After buffering with potassium dihydrogen phosphate (0.1 M), the tissues were subjected to further digestion with proteinase-K (50 mg/ml) (Merck, Germany) digestion at 37°C with intermittent shaking. The content was mixed vigorously with phenol (Phenol saturated tris-EDTA; TE, Wako Pure Chemical Industries, Japan) and centrifuged at 3,000 rpm for 15 minutes using a cold centrifuge (MR 150, Tomy Seiko Co Ltd, Japan). The super-natant thus recovered was mixed with chloroform (24:1 mixture of chloroform and isoamyl alcohol) followed by centrifugation at 3,000 rpm for 15 minutes. The resulting supernatant was subjected to ethanol precipitation at -30°C and centrifuged at 14,000 rpm for 15 minutes. After washing in 70% ethanol, the DNA pellet was allowed to dry and was dissolved in 100 ml of TE buffer (1 mM EDTA in 10 mM Tris-HCl, pH 7.4). The biopsy sized liver tissue (measuring about 2 mm^3 in size) was placed into a micro centrifuge tube, minced with plastic stick, suspended in SE buffer, and processed as the frozen liver tissues.

Primers

The *Toxocara* primers used in this study were: 5'-AGCCGAAAGTGTATCAAGGA-3' (SB12 forward) and 5'-TGATGTTCTTGCCGCTGTTA-3' (SB12 reverse) with product length of 294 base pair (bp).

PCR

DNA extract (1.5 ml), PCR mix (10.44 ml) (Takara Co, Japan), Taq polymerase (0.06 ml) (Takara Co, Japan), primer SB12 forward (1.5 ml) and primer SB12 reverse (1.5 ml) were mixed into a PCR tube (Eppendorf, Germany), overlayed with a drop of mineral oil (Sigma Chemicals Co, USA) and subjected to a 30 cycle PCR with a profile of 92°C for 30 seconds, 51°C for 30 seconds, and 72°C for 60 seconds (Zymoreactor II AB 1820; Atto Corporation, Japan).

PCR product electrophoresis

The PCR products were resolved by electrophoresis (Mupid 02Z1, Advance Co Ltd, Japan) in 1.5% agarose (FMC Bio Products, USA) gel and Tris-Borate-EDTA buffer with added ethidium bromide at 100 V for 45 minutes along with molecular weight markers (Pharmacia, USA). The electrophoretic bands were visualized under UV light and photographed.

Second PCR

Second PCR was performed using the first PCR product as template DNA and visualized as described above.

RESULTS

In this study, first PCR revealed positive bands with the expected number of base pairs in biopsy sized liver tissues of 1,000 and 10,000 eggs infected mouse liver, in remaining liver tissue of 1,000 eggs infected mice, and in both *T. canis* and *T. cati* eggs (Fig 1). However, on second PCR (using the PCR product as template DNA), all biopsy sized and remaining liver tissue samples were found to be

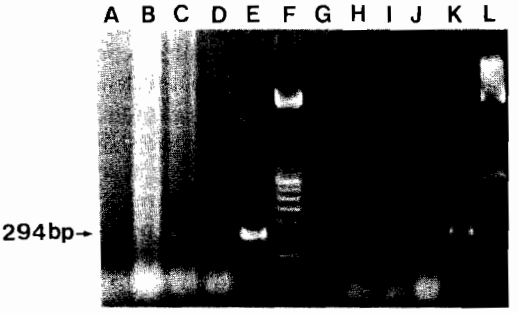

Fig 1–Result of first *Toxocara* PCR. Lanes: A to D, biopsy size liver tissues (A: 10, B: 100, C: 1,000 and D: 10,000 *Toxocara* eggs infected mice); G to J, remaining mouse liver tissues of same mice (G: 10, H: 100, I: 1,000 and J: 10,000 eggs infected); E, *T. canis* eggs; K, *T. cati* eggs and F and L, molecular markers.

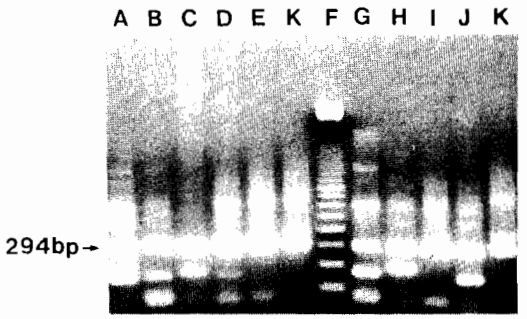

Fig 2–Results of second PCR. Lanes: A to D, biopsy size mouse liver tissues (A: 10, B: 100, C: 1,000 and D: 10,000 *Toxocara* eggs infected mice); G to J, remaining mouse liver tissues of same mice (G: 10, H: 100, I: 1,000 and J: 10,000 eggs infected); E, *T. canis* eggs; K, *T. cati* eggs and F, molecular markers.

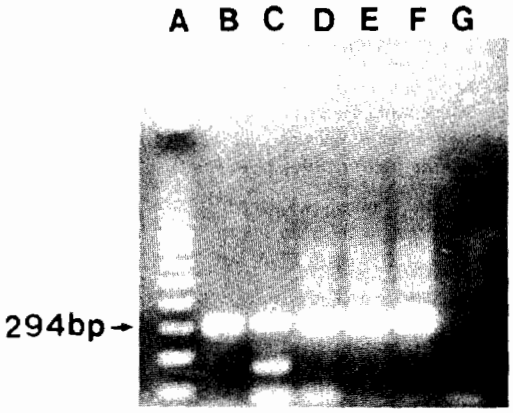

Fig 3–Results of second PCR. Lanes: A, molecular markers; B to E, Biopsy size liver tissues (second lot) (B: 10, C: 100, D: 1,000 and E: 10,000 *Toxocara* eggs infected mice); F, *T. canis* eggs and G, *A. suum* eggs.

positive (Fig 2). No positive band was shown by *A. suum* eggs even on the second PCR (Fig 3). Positive bands in *T. canis* and *T. cati* eggs and no band in *A. suum* eggs indicated the specificity of the primers (SB12) for *Toxocara* (Fig 3). On the second PCR, the bands were sufficiently large and correlated well with the infective dose of eggs. Good reproducibility of double PCR was observed. Some non-specific extra bands, however, were also visualized in second PCR results (Fig 2). Repeat experiments showed similar results, indicating good reproducibility.

DISCUSSION

Use of molecular techniques in the study of medically important helminths has been increasing. These includes *Echinococcus* sp (Bretagne *et al*, 1993; Yagi and Ohyama, 1994; Martin *et al*, 1995), *Taenia* sp (Chapman *et al*, 1995; Gottstein *et al*, 1991; Bowles and McManus, 1994), *Trichinella* sp (Bandi *et al*, 1993; Takahashi *et al*, 1995), *Fasciola hepatica* (Rognlie *et al*, 1995), *Schistosoma* sp (Dias Neto *et al*, 1993; Zhang *et al*, 1995), and some filarial worms (Yenbuter and Scott, 1995). However, its use in the detection of helminthic parasites in clinical sample is limited. To the best of our knowledge, no reports on the use of PCR technic in the study of *Toxocara* parasites or in the diagnosis of toxocariasis were available to date. For the first time we report the use of PCR technique in the diagnosis of VLM in experimentally infected mice using primers developed at one of our laboratory recently.

In the present study, we evaluated the usefulness of the PCR technique in the diagnosis of VLM and also tested the specificity of the primers developed recently at one of our laboratory. For this purpose, we performed PCR on liver samples recovered from different dose-specified embryonated *T. canis* egg infected mice. We could detect the migrating larva successfully in the mouse liver infected by as few as 10 embryonated eggs by means of double PCR. In case of heavy infection, however, the first PCR appeared good enough to detect the parasite in liver sample. Keeping in view of human cases of VLM, we tested the biopsy sized liver tissue of the same mouse liver separately to see the applicability of PCR technic in liver biopsy materials taken from a patient with VLM. All biopsy-size liver tissues taken from four different dose of embryonated eggs infected mice were positive. This finding suggested the applicability of PCR technic in the diagnosis of VLM when required and appears to be helpful particularly in areas having high *Toxocara* seroprevalence.

On second PCR, though the specific bands were prominent, some extra non-specific bands were also visualized. The appearance of extra bands on second PCR could be due to the amplification of minor bands of repetitive sequences of parasitic DNA which have undergone point mutation. However, our present findings were highly promising in the diagnosis of VLM by PCR method with the use of primers (SB12).

There was no bands will *A. suum* eggs indicating that the primers SB 12 were specific for *Toxo-cara* sp. . These primers were found to be very good diagnostic tools for the diagnosis of VLM caused by *Toxocara* sp (*T. canis* and *T. cati*). Since these primers were genus specific, we are now in the process of developing species specific primers with the aim to identify *T. canis* and *T. cati* separately as has been reported for *Taenia* sp (Gottstein *et al*, 1991; Chapman *et al*, 1995) and for *Schistosoma* sp (Dias Neto *et al*, 1993) and *Echinostoma* sp (Fujino *et al*, 1995) by arbitarily amplified PCR. However, species specific primers are not required so far as the diagnosis of VLM is concerned as both species of *Toxocara* cause the VLM in man.

In this study, it was evident that the primers we used were specific to *Toxocara* and were useful in the diagnosis of VLM provided liver biopsy materials were available from a suspected case of VLM. The PCR technic, however, needs to be evaluated in human cases.

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