Archives of Razi Institute, Vol. 63, No. 2, December (2008) 35-40

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Detection of *Ornithobilharzia turkestanikum* cercaria (trematoda) by nested-PCR in intermediate host snail, *Lymnaea gedrosiana*

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Received 02 May 2008; accepted 21 Oct 2008

ABSTRACT

Trematodes are important in economic and public health. *Ornithobilharzia turkestanicum* (O. *turkestanicum*) is one of the important economic trematodes in domestic animals. *Ornithobilharzia* infection in intermediate host (*Lymnaea gedrosiana*) can be detected by either exposing snails to light to induce cercarial shedding or by squeezing them between glass slides to detect parasites. The current available diagnostic methods are inefficient for identification of prepatent infections and/or after dead of snails. For the above difficulties we adapted a nested polymerase chain reaction (Nested-PCR) assay for sensitive detection of O. *turkestanicum* in clinical samples and its cercaria in snails. The life cycle of parasite was maintained in sheep and snails in laboratory in Razi Institute. Adult worms were isolated from sheep and DNA was extracted from worms by a procedure using DNA extraction solution developed in NIGEB. PCR and nested-PCR primers were designed based on 28s ribosomal RNA gene of *O. turkestanicum* and the DNA was amplified by PCR assay. PCR product was purified and cloned in pTZ57R/T and sequenced. The comparison of the obtained sequences with the GenBank using blast program was showed in NCBI Sequence viewer just 682 bp. PCR amplicon was submitted in GenBank and can be assessed using AY862391 accession number. DNA was extracted from the infected and non-infected snails 2-5 days post-infection. The infected snails could be rapidly detected with Nested-PCR. Results indicate that this assay is specific for detecting *O. turkestanicum.* The high sensitivity of the test enabled identification of single infected snail even when its DNA was pooled with uninfected snails. Thus demonstrating the possibility of mass diagnosis in pools of snails, therefore, the assay has the potential for large-scale demonstration of prepatent infection prevalence in snails and offers a new diagnostic tool for evaluation of bilharziosis trasnsmission and for control of infection as discussed.

Keywords: PCR, Nested-PCR, Diagnosis, Trematoda, *Ornithobilharzia turkestanicum*

INTRODUCTION

Schistosome blood flukes live in the blood vessels of man and animals. *Schistosoma turkestanicum* was first described by Skrjabin (1913) from portal vein in cattle of Russian Turkestan (Machattie 1936). Price (1929) points out that the egg of this Schistosome had two spines, one at each end and the number of testes. Adult worm were detected

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chiefly in mesenteric veins. He transferred it to the genus *Ornithobilharzia* Odhner, 1912. Dutt and Srivastava (1955) suggested transferring to a new genus called *Orientobilharzia* and other names but have not found general acceptance. *O. turkestanicum* has been recorded in cats,cattle, sheep, goats, water buffaloes, horses, donkeys, mules, camels (Soulsby 1982). The male and female size are 2-10 mm and 2-8mm respectively. Man appears to be immune. Dogs, ducks, geese and other birds showed no trace of infection. *O. turkestanicum* appears to cause very harmful effects on the sheep and goats. The liver portal system and intestinal mucous memberane are chiefly affected. The total economic loss is great (Machattie 1936). *O. turkestanicum* was reported from Khozestan,Iran by Dezful Bilharziasis Pilot Project was done in 1962, and later was reported in large animals (Massoud 1974). It is a dangerous parasite of domestic animals in the marsh and rice field areas in Iran. The arrest of the egg in the intestinal mucosa and the consequent formation of nodules largely destroys the value of the intestine as a commercial product. A thorough investigation of the molluscan fauna was revealed that the most prevalent snail as intermediate host were lymnaea species. *O. turkestanicum* and the other related schistosoma are distributed widely throughout some provinces of Iran (Eslami 1990). Because intermediate hosts of these species: 1) may be found in the same freshwater habitat and 2) have morphologically similar cercaria, better means are needed to tell them apart. Infected Snails and contact patterns of susceptible animals with inoculated water with cercaria of parasite are important factors in transmission of *Ornithobilharzia* (Hamburger *et al* 1998). For diagnosis of parasite, fecal examination of animals and snails infection determined by searching for eggs and cercaria respectively, are suitable methods. But, these methods are time-consuming, and labour intensive when large scale samples have to be examined. These techniques can be also inaccurate in chronic disease situations or when snails mortality is high. The aims of this study was to give more information about the *O. turkestanicum*, to developed a polymerase chain reaction (PCR) and Nested-PCR assays for identifying *O. turkestanicum* and larvae of the trematods in intermediate host in prepatent period and detection of cercaria of parasite before reaching to infectious stage for the first time in Iran in clinical samples as a rapid and reliable diagnostic assay and to improve update diagnostic test for *O. turkestanicum* in laboratory.

MATERIALS AND METHODS

Young laboratory bred *Lymnaea gedrosiana* were infected with miracidia of *O. turkestanicum* cultured from intestinal feces of naturally infected sheep. Briefly, snails were collected from natural habitat and transferred to parasitology laboratory of Razi Institute. They gradually adapted themselves to the artificial environment. The *O. turkestanicum* (Iranian strain) eggs (Figure 1) were collected from naturally infected sheep and they were cultured for miracidia production. *Lymnaea gedrosiana* were infected with miracidia (Figure 2) of *O. turkestanicum* hatched from the eggs collected and cultured in laboratory.

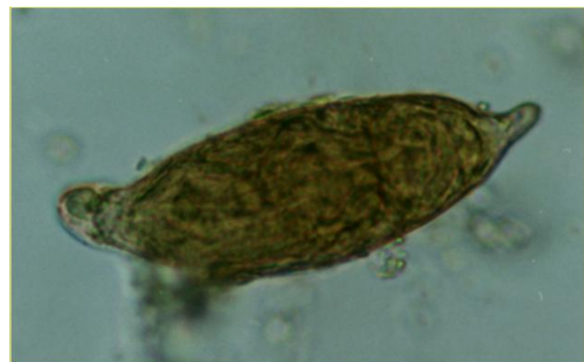

Figure 1. Parasite egg.

The snails were exposed to miracidia. The miracidia were counted carefully under a dissecting

microscope and transferred with a fine pippet to a plastic tank (Figure 3).

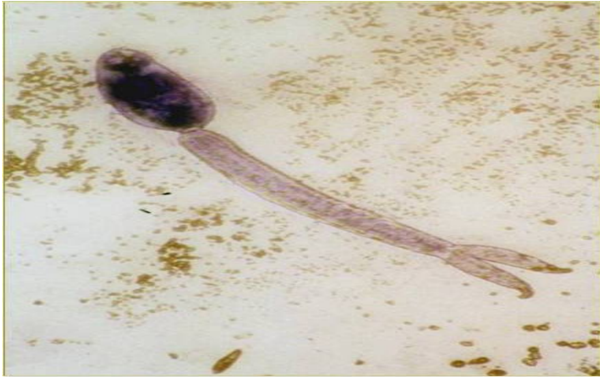

Figure 2. Cercaria.

Figure 3. Plastic dish containing Snails.

Then the snails were divided in groups and maintained in separate plastic tanks at 24ºc -28ºc (Massoud 1973, Machattie 1936). The snails from day 15 after exposure were examined for cercaria shedding in a peteri dish. Snails were found to begin shedding cercaria on days 18-21 after exposure to miracidium **.** Sheep aged 6-8 months, were infected. Each animal was exposed to 5000 cercaria of O. *turkestanicum*. by the oral technique, and was autopsied 12 or more weeks after exposure (Massoud 1974). Faecal examination was begun 30 days after exposure to the cercaria. Worms were recovered from mesenteric veins (Figure 4). Examinations of slaughtered animals were carried out by transilluminating the mesenteric veins with a strong direct light (Massoud 1974, Machattie 1936).

Preparation of DNA from worms. Adult worms were collected from the mesentric veins of infected sheep. Adult worms were homogenized in one ml of distilled water. Mesentric tissue containing adult worm was also homogenized.

Figure 4. Adult male and female parasites.

Mesenteric tissue of healthy sheep was used as negative control. *Fasciola* and *Dicrocelium* (members of trematoda class) were used also as controls. Infected and non-infected snails were also homogenized and DNA was extracted from one individual or pooled samples (Janotti passos 1997). Total DNA was extracted from described specimens by a procedure using DNA extraction solution (developed in NIGEB). Briefly, in a 1.5ml tube, 200µl of homogenate was added to 900µl of extraction solution. The sample was vortexed and 400µl chloroform was added. After centerifugation (12000xg for 15 min), aqueous phase was transferred to a new tube and equal volume of cold isopropanol was added. The sample was incubated 15 min on ice and DNA was precipitated by 12000xg centrifugation. The pellet was washed with 70% ethanol, dried in air and resuspended in 20 µl dH_2O. The DNA concentration and purity was measured with a spectrophotometer. The extracted DNA was kept at -20 ºC until used (Hamburger *et al* 1998).

Designing of primers. PCR primers were selected from 28S ribosomal RNA genes of avian, domestic animals Schistosoma and O. *turkestanicum* published sequences in GenBank. The nucleotide and sequences positions of primers are as follows, *O. turkestanicum* -F, 5`-CAC CAA TGA GTA CGC CTT CCC -3` and *O. turkestanicum* -R, 5`-CAG ACA CGG AAG TGG CAA CGC -3`.

Amplification of DNA in PCR and nested-PCR. Amplification of DNA was performed in a total volume of 50 µl containing 2 µl of DNA template(0.5-2 µl) from each specimen,1.5 mM $MgCl_2$, 250 ng of each primer, 5 µl of 10xPCR reaction buffer, 0.02 mM of dNTPs and 0. 2 U of Taq DNA polymerase and sterile water to bring the final volume up to 50 µl. The amplification was carried out in a DNA Thermalcycler (Techne, England): the reaction mixtures were initially heated at 94 ºC for 3min (one cycle), and were then subjected to 30 cycles at 94 ºC (30 seconds) for denaturation, 52 ºC, (30 seconds) for primer annealing and 72 ºC, (30 seconds) for amplicon extension (Hamburger *et a*l 1998, Yiping *et a*l 1998). Finally an elongation step at 72 ºC for 10 min was included. Optimization of PCR was carried out using positive and negative controls (Figures 5, 6).

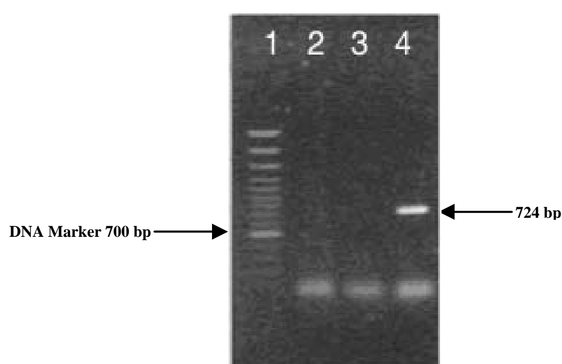

Figure 5. Differential diagnosis of *O. turkestanicum.* by PCR.
Lane 1: DNA size marker (100 bp Ladder)
Lane 2: Dicrocelium
Lane 3: Fasciola
Lane 4: infected snail with larvae of *O. turkestanicum.*

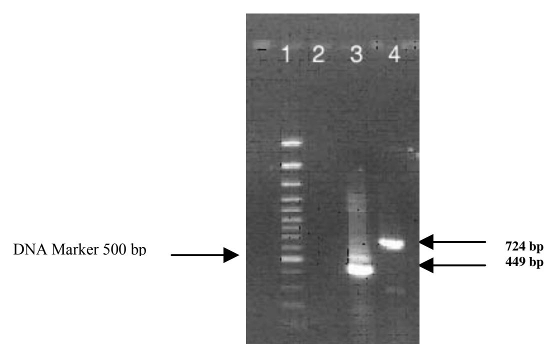

Figure 6. Detection of *O.turkestanicum.* in infected tissue in 1% agarose gel (PCR andNested-PCR)
Lane 1: DNA size marker (100 bp Ladder)
Lane 2: Uninfected tissue(negative control)
Lane 3: Nested-PCR of infected tissue(449bp)
Lane 4: PCR of infected tissue(724bp)

Cloning and sequencing of PCR product. The PCR product from *O. turkestanicum* sample was purified using High Pure PCR product Purification Kit (Roche, Germany), cloned in a pTZ57R/T (Fermentas, Lithuania) and sequenced. The comparison of the obtained sequences with the GenBank was performed using Blast program (Sambrook *et al* 1989).

Nested-PCR. Nested primers were designed from the sequence data of PCR products. The sequence of nested primers is as follows:

O. turkestanicum Nested-F: 5′-GTC GCG TTG TTT GTG AAT GC-3′ and *O. turkestanicum*-Nested-R: 5′- ATG AGC GAA AGA GTC ACC TG-3′. Nested-PCR was performed with a 1:500 dilution of the end products of PCR. After amplification each samples were analysed by agarose gel electrophoresis in TBE buffer pH 8.0. The gels were stained for 20min with ethidium bromide (0.5 µg/ml) and washed for 10 min in double-distilled water on a shaker, viewed on a UV transilluminator and photographed (Hamburger *et al* 1998, Hanelt *et al* 1977, Yiping *et al* 1998).

RESULTS

Snails were found to begin shedding cercaria on days 18-21 after exposed to miracidium**.** Worms

were recovered from mesenteric veins of sheep on 6-7 weeks after they were infected.

For sensitive detection of *O. turkestanicum* by molecular assays in infected hosts and its cercaria in intermediate host snails, primers were designed based on 28s rRNA gene of parasite and the DNA was amplified by PCR and Nested-PCR. DNA amplification of parasite and infected snails were showed about 724 and 449 bp products in agarose gel respectively. As shown in figuers: 5 and 6, an expected size of 724 bp product unique for *O. turkestanicum* was amplified. The target band was detectable if at least one worm was present in the sample, however, PCR provided a more intensive DNA band if more than one worm was present in the sample. No DNA bands from uninfected snail, control tissue, *Fasciola* and *Dicrocoelium* (class: trematoda) samples were amplified when *O. turkestanicum* specific primers used. Mesentric tissue sample containing at least one *O .turkestanicum* worm produced the expected size PCR product, while negative control tissue sample failed to produce any DNA band (Figure 6). The PCR product from *O. turkestanicum* sample was purified and sequenced. A 1:500 proportion of DNA from an infected snail by 2 miracidia produced 449 bp product in Nested-PCR (Figure 6). The nucleotide sequence of Comparison of PCR product sequence with 28S ribosomal RNA of *O. turkestanicum* in the Gen Bank was found identical. Among 20 snails exposed to 2-4 miracidia, 85% were PCR positive one week later. 20 snails each exposed to 2-4 miracidia was left for six weeks, and shedding cercaria (all 20 snails) carried out. These results showed that the sensitivity of PCR and Nested-PCR is 100%. The comparison of the obtained sequences with the GenBank using blast program was showed in NCBI Sequence viewer just 682 bp. PCR amplicon was submitted in GenBank and can be assessed using AY862391 accession number. Identification of an infected snail when its DNA is polled with DNA from several uninfected snails may increase the practicability of using of PCR and Nested-PCR assay for mass screening of prepatent infections. In our experiments the detection limit was reached when DNA from one infected snail was diluted with 50 equal aliquots of DNA from uninfected snails.

DISCUSSION

The prevalence of *O. turkestanicum* in sheep and goats is reported as 35% and 100% in provinces of Iran (Eslami 1990). Infection rates in field populations of snails are routinely determined by searching for cercaria shed from snails with patent infection (Massoud 1973). In contrast, prepatent infections, which can constitute a significant proportion of snails populations, are not determined routinely because of a lack of a suitable method (Hamburger *et al* 1998). The PCR assay described in the present study was based on amplification of a part of 28S rRNA gene sequence of the *O. turkestanicum* parasite. This assay was shown to be specific for detecting *O. turkestanicum* rather than other trematods, but further specificity analyses are required by cross-amplification assay with DNA from a variety of trematods (Scott *et a*l 1998). Remarkably little is known about *O. turkestanicum* as a parasitic infection. However according to some clinical or laboratory observations the prevalence of this infection could be high in Iran (Eslami 1990, Hosseini 1997). These data were published by PCR test about *O. turkestanicum* for the first time in Iran. Commonly, identification of *O. turkestanicum* in infected animals performed by periodical examination of the animals' feces and detection of parasite egg. However when the infection is in chronic phase the detection of egg would not be easy. Other methods for diagnosis of parasite is dissecting veins and separating parasite, then treated with clearing agent and staining for visualizing its internal organs to differentiate from other similar schistosomes. Another approach is to

examine snails which are maintained for several weeks in the laboratory for shedding cercaria. However these methods are time consuming and labour intensive therefore may not be practiced routinely on a large scale and also can be highly inaccurate. To date very little information is available concerning the diagnosis of the *O. turkestanicum* infection and parasite itself. Lack of serological and immunological assay also has caused little available research data and reports (Janotti Passos 1997). Due to above difficulties and since the monitoring of parasite in animals is difficult and inaccurate the simple PCR procedure developed in this study for monitoring infected animals would facilitated the diagnosis of *O. turkestanicum* worms. A small segment of peritoneal tissue should facilitate the PCR approach. Since the collection of peritoneal tissue after slaughter is simple and can be done by veterinary technicians, slaughter house workers and butchers, one can make a convenient separation of peritoneal segments collection, storage and carry for their testing by PCR in central laboratories. The PCR in pools of peritoneal material should greatly facilitate large scale survey for determination of the prevalence of *O. turkestanicum* in susceptible animal populations. The sensitivity of this assay was demonstrated by the detection of one worm or one inoculated snail. In the present study the PCR and Nested-PCR assay identified infected snails at 5days and one week after they were exposed to 2-4 miracidia.

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