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Original Article

Genetic Identification of *Orientobilharzia turkestanicum* from Sheep Isolates in Iran

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Abstract

Background: Adult worms of *Orientobilharzia turkestanicum* live in the portal veins, or intestinal veins of cattle, sheep, goat and many other mammals causing orientobilharziasis. Orientobilharziasis causes significant economic losses to livestock industry of Iran. However, there is limited information about genotypes of *O. turkestanicum* in Iran.

Methods: In this study, 30 isolates of *O. turkestanicum* obtained from sheep were characterized by sequencing mitochondrial cytochrome c oxidase subunit 1 (cox1) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (nad1) gene. The mitochondrial cox1 and nad1 DNA were amplified by polymerase chain reaction (PCR) and then sequenced and compared with *O. turkestanicum* and that of other members of the Schistosomatidae available in GenBank™.

Results: Phylogenetic relationships between them were re-constructed using the maximum parsimony method. Phylogenetic analyses done in present study placed *O. turkestanicum* within the Schistosoma genus, and indicates that *O. turkestanicum* was phylogenetically closer to the African schistosome group than to the Asian schistosome group.

Conclusion: Comparison of nad1 and cox1 sequences of *O. turkestanicum* obtained in this study with corresponding sequences available in Genbank™ revealed some sequence variations and provided evidence for presence of microvariants in Iran.

Introduction

Parasites of the genus *Orientobilharzia* belong to Platyhelminthes, Trematoda, Digenea, Schistosomatidae, and the type species is *Orientobilharzia turkestanicum* (1, 2). Adult worms of *O. turkestanicum* live in portal veins, lungs or intestinal veins of cattle, sheep, goat and large numbers of other mammals (5, 6). The body size of male and female worm is 2-10 mm and 2-8 mm respectively (7). This parasite causes orientobilharziasis infection (6) reported in many parts of the world such as Kazakhstan, China, Pakistan, Mongolia, India, Iraq and Iran in Asia, and Russia and Turkey in Europe (2, 3, 4, 7,8).

Despite animals such as dogs and birds, which did not show any sign of infection, this parasite causes harmful effects on venous systems and mucous membranes in sheep and goat (9). Cattle and sheep that are infected with *O. turkestanicum* often display a chronic disease, presenting the syndrome of emaciation, anemia and diarrhea, and may have deciduous mucosa and blood in the feces. Other symptoms include pale mucous membranes, edema in the jaw and abdomen. The female animals may suffer from abortion, and the young animals grow slowly (10). More importantly, the cercariae of *O. turkestanicum* can also infect humans and often cause cercarial dermatitis (11) and are considered the major pathogen of cercarial dermatitis in the Caspian Sea area of Iran (12) and several provinces of the People's Republic of China (6) posing significant public health problem in a number of countries (10). According to previous studies many species of *Orientobilharzia* have been reported named *O. bomfordi* (13), *O. cheni* (14), *O. turkestanicum* (2), *O. dattai* (15), *O. harinasutai* (16) and *O. turkestanicum var. tuberculata* (17). Traditional classification and understanding of phylogenetic relationships among Schistosomatidae is based on geographic distribution, morphological characteristics of adult parasites and eggs, transmission and pathogenesis

as well. Today, it has becoming a common practice to use DNA sequencing for inferring evolutionary relationship among parasites.

The present study was done to sequence the mitochondrial cytochrome c oxidase subunit1 and nicotinamide adenine dinucleotide dehydrogenase subunit1 genes of *O. turkestanicum* from sheep isolates in Iran and compare them with corresponding sequences of *Orientobilharzia* spp., available in GenBank™ for any probable variations and examine the phylogenetic relationships of *O. turkestanicum* with other members of the Schistosomatidae using the nucleotide sequences of *cox1* and *nad1* mtDNA.

Materials and Methods

Specimen collection

The *O. turkestanicum* isolate used in present study was from Mazandaran Province in Iran, 2014. Adult blood fluke of *O. turkestanicum* was obtained from the portal and mesenteric veins of sheep (Fig. 1), washed extensively in physiological saline, identified based on morphological characters according to existing keys and descriptions (18), and fixed in 70% (v/v) ethanol until use. For confirmation of diagnosis, 5 trematodes fixed in alcohol 70 % with a little glycerin were sent to British Natural Museum. The specimen deposited in British Natural Museum as *O. turkestanicum* with the code of BM (2013.04.18.1-5).

DNA extraction and amplification

Total genomic DNA was extracted from a mixture of five males and five females by QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN). The DNA sample was stored at -20 °C until further use. To obtain mtDNA sequences, fragments of approximately 1,100 bp and 525 bp of mitochondrial cytochrome C oxidase subunit 1 (*cox1*) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) genes were amplified respectively using forward primer p1

(5'- GGCGGGTTTATAGGTTTAG -3') and altered reverse primer p2 (5'- CTTATTTAA-TGAATAACCAACTATA - 3') for cox1 gene reported by Li, 2008 and forward primer p3 (5'-

AGATTCGTAAGGGGCCTAATA -3') and reverse primer p4 (5'- ACCACTAACT-AATTTCAC-TTTCGGA -3')for nad1 gene (Li, 2008).

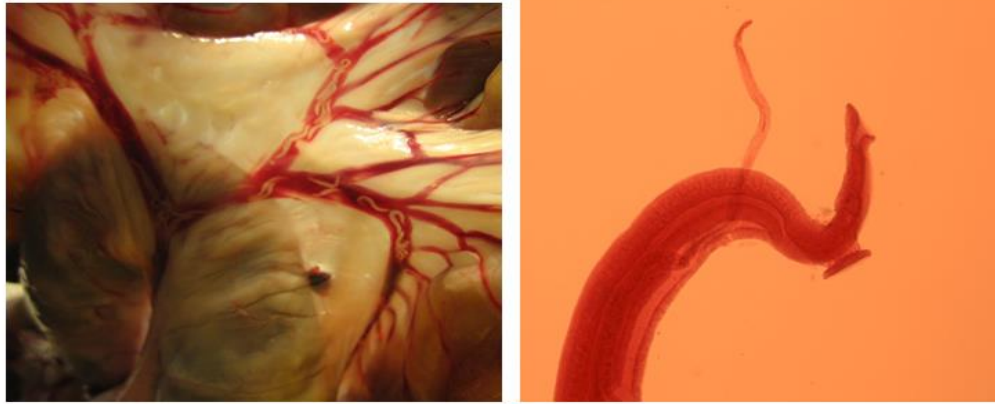


Fig. 1: *Orientobilharzia turkestanicum* in mesenteric veins in a sheep Infected (left) and Adult male & Female of *Orientobilharzia turkestanicum* isolated from infected sheep (right)

The PCR mixture was carried out in 50 μ l volumes containing 2 μ l of sample DNA (100 ng), 1 \times PCR buffer, 2 mM MgCl₂, 240 μ M dNTP mix 200 nM of each primers and 1.5 unit Taq DNA polymerase, in an automated thermocycler. The PCR was performed using the following protocol: 3 minutes incubation at 94 °C to denature the double stranded DNA, then 35 cycles of a 1 min at 94 °C (denaturing step), 45 s at 53 °C (annealing step), and 45 s at 72 °C (extension step). Finally, the PCR was completed with an additional extension step for 10 min for cox1. For nad1, PCR protocol was as 3 minutes incubation at 94 °C, then 35 cycles of a 1 min at 94 °C (denaturing step), 45 s at 50 °C (annealing step), and 45 s at 72 °C (extension step), followed by final extension at 72 °C for 10 min. Samples without genomic DNA were used as negative controls. The PCR products were analyzed on 1.5% agarose gels in 0.5 \times TBE buffer and visualized using gel documentation system (KODAK Gel Logic 200 Imaging System).

Sequence alignment and Phylogenetic analyses

The PCR product was purified using a quick PCR product purification kit (Roche) according to the manufacturer's recommendations. DNA sequencing was performed for each of the PCR products by Bioneer Company. The sequence chromatograms were analyzed using the Chromas version 3.1 software and compared to those registered in GenBank™ using the 'Basic Local Alignment Search Tool' (BLAST). Then the nucleotide sequences were aligned using the ClstalW method of MegAlign (DNA Star) and Mega5 programs. Phylogenetic analyses were performed using the maximum parsimony method utilizing the Mega5 program. A correspondent nucleotide sequence of *Fasciola hepatica* (GenBank™ accession AJ628039 for cox1 gene and AJ630405 for nad1 gene) was used as an out-group.

Results

The extracted genomic DNA was used to amplify the cox1 and nad 1 PCR. The cox1 and nad1 PCR products showed an expected

fragment of nearly 1,100 bp and 500 bp in length respectively. Nucleotide sequence analysis revealed that all of the samples were *Orientobilharzia turkestanicum*. The sequences were analyzed by multiple alignments with reported

reference sequences for the *Orientobilharzia* genotype.

The phylogenetic relationship between the isolated sequences and various genotypes of orientobilharzia based on *cox1* and *nad1* genes were shown in Fig. 2.

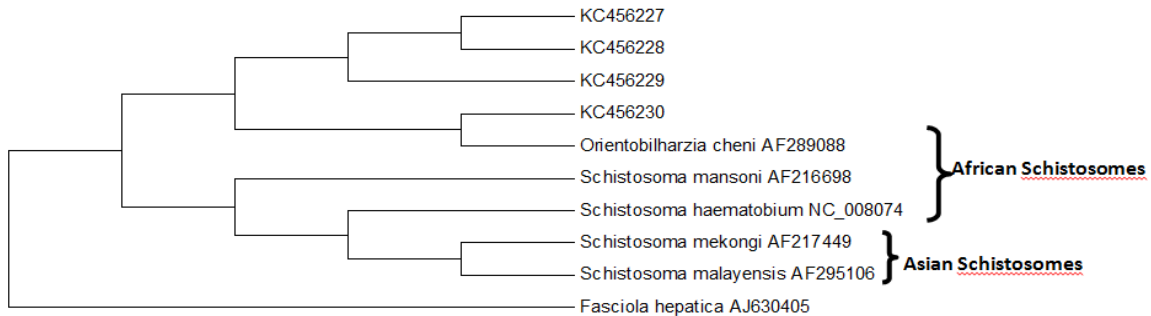


Fig.2-A: phylogenetic relationship between the isolated sequences and various members of Schistosomatidae by maximum parsimony method based on the *nad1* sequences. *Fasciola hepatica* (AJ630405) used as the out group. The various members of the Schistosomatidae used were consist of: *orientobilharzia cheni* (AF289088), *Schistosoma haematobium* (NC_008074), *Schistosoma mansoni* (AF216698), *Schistosoma malayensis* (AF295106) and *Schistosoma mekongi* (AF217449)

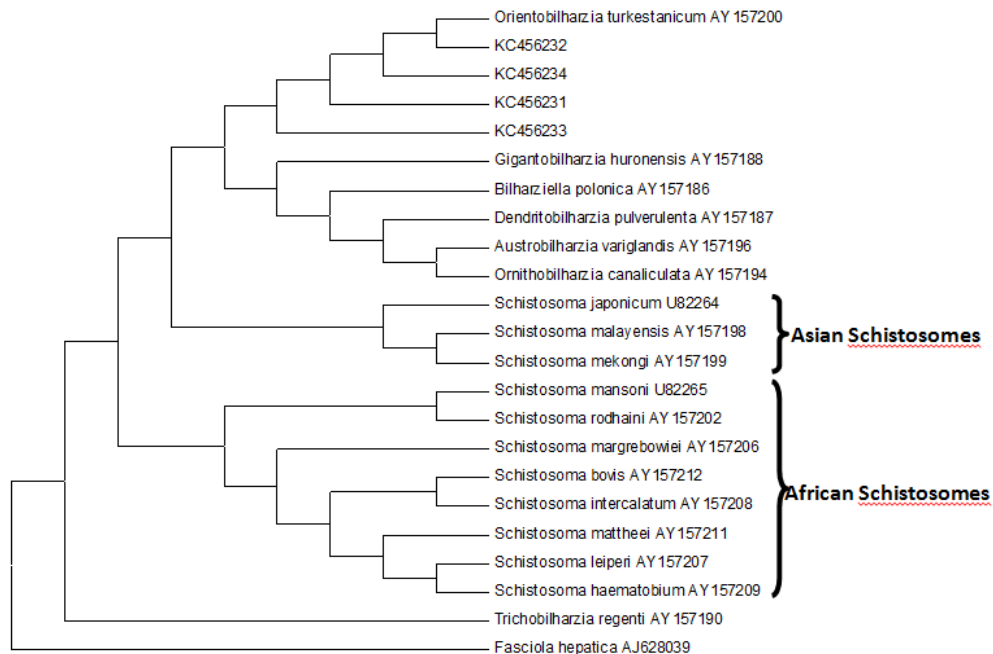


Fig. 2-B: phylogenetic relationship between the isolated sequences and various members of Schistosomatidae by maximum parsimony method based on the *cox1* sequences. *Fasciola hepatica* (AJ628039) used as the out group. The various members of the Schistosomatidae that used were consist of *Schistosoma bovis* (AY157212), *Schistosoma intercalatum* (AY157208), *Schistosoma leiperi* (AY157207), *Schistosoma haematobium* (AY157209), *Schistosoma mattheei* (AY157211), *Schistosoma margrebowiei* (AY157206), *Schistosoma mansoni* (U82265), *Schistosoma rodhaini* (AY157202), *Orientobilharzia turkestanicum* (AY157200), *Schistosoma malayensis* (AY157198), *Schistosoma mekongi* (AY157199), *Schistosoma japonicum* (U82264), *Gigantobilharzia huronensis* (AY157188), *Trichobilharzia regenti* (AY157190), *Dendritobilharzia pulverulenta* (AY157187), *Bilharziella polonica* (AY157186), *Austroilharzia variglandis* (AY157196), *Orientobilharzia canaliculata* (AY157194)

Phylogenetic analyses done in present study placed *O. turkestanicum* within the *Schistosoma* genus, and indicates that *O. turkestanicum* was phylogenetically closer to the African schistosome group than to the Asian schistosome group. The sequencing results of *cox1* and *nad1* genes of *O. turkestanicum* from sheep isolates of Iran were registered in GenBank™ under the following accession numbers: KC456231, KC456232, KC456233, KC456234 for *cox1* and KC456227, KC456228, KC456229, KC456230 for *nad1*.

Discussion

Limited surveys have been performed on the genotypes of *O. turkestanicum* originating from sheep around the world, and the present study is the first comprehensive strain characterization of sheep isolates in Iran. *O. turkestanicum* was reported from Khuzestan Province of Iran (19). *O. turkestanicum* and some other related *Schistosoma* are distributed throughout some regions of Iran (20) and in a latest study which was performed by Razi Vaccine and Serum Research Institute, *O. turkestanicum* cercaria was detected by nested-PCR in intermediate snail, in Iran (9). The present study is the first genotype analysis of *O. turkestanicum* from sheep isolates in Iran. Mitochondrial genome is one of the best targets for discriminating between strains, so *cox1* and *nad1* genes provided valuable information for detection of variants of *Orientobilharzia* (6) as present study used *nad1* and *cox1* mitochondrial genes for molecular characterization of *O. turkestanicum*. Traditional classification of schistosomes based on different factors such as geographical distributions, intermediate and definitive hosts and morphological characteristics of adult parasites and eggs (10) is not a reliable method for systematic studies of schistosomes. So, DNA sequences of mitochondrial genes can be used as genetic markers for reliable systematic studies (21). Comparison of

nad1 sequences of *O. turkestanicum* obtained in the present study with corresponding sequences available in Genbank™ (Accession number AF289088) revealed that there were few sequence variations at sequence position 112 (T to A) for KC456227, sequence position 276 (T to C) for KC456227, KC456228, KC456229 and KC456230, sequence position 327 (G to A) for KC456227, KC456228 and KC456229. Also comparison of the *cox1* representing the *O. turkestanicum* samples examined in the present study with the corresponding sequence available in Genbank™ (Accession number AY157200) revealed that there were also sequence variations at sequence positions 196 (G to A), 233 (G to A) and 441 (G to A) for KC456233, sequence positions 233 (G to C) and 325 (G to A) for KC456232 and sequence position 233 (G to A) for KC456234. These variations showed that *O. turkestanicum* parasite like *Echinococcus granulosus* genotypes G1 may have microvariants as reported in Iran (22), Prue (23) and Turkey (24).

Phylogenetic relationship of the *schistosomes* has been the focus of several recent studies using different genetic makers and approaches (25-29), and some of these studies placed *O. turkestanicum* within the genus *Schistosoma*. In the present study, the partial *cox1* and *nad1* DNA of *O. turkestanicum* were amplified and sequenced used to re-construct the phylogenetic relationships of *O. turkestanicum* with other members of the Schistosomatidae using maximum parsimony method.

Phylogenetic analyses performed in present study placed *O. turkestanicum* within the *Schistosoma* genus, and indicates that *O. turkestanicum* was phylogenetically closer to the African schistosome group than to the Asian schistosome group; it was in common with the study of Wang et al. for the phylogenetic position of *O. turkestanicum* within the family of Schistosomatidae (10). Though *Orientobilharzia* species are now considered as important zoonotic agents, studies on their biochemistry, histochemistry, immunopathology and molec-

ular biology are lacking and limited compare was performed to *S. japonicum* and *S. mansoni*. *Orientobilharziasis* is an important and severe disease in several mammals, and cercarial dermatitis caused by *Orientobilharzia* spp. may be regarded as an under-reported/neglected disease. Therefore, it is important to evaluate in detail the impact of *Orientobilharzia* species on human and animal health from a global perspective and intensive studies are needed in order to better control of human and animal infections with *Orientobilharzia* species (10). The present study is the first comprehensive genotypic analysis of *O. turkestanicum* infecting sheep by using PCR analysis and DNA sequencing in Iran and provided evidence for presence of microvariants.

Conclusion

The present study is the first comprehensive genotypic analysis of *O. turkestanicum* infecting sheep by using PCR analysis and DNA sequencing in Iran and provided evidence for presence of microvariants.

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