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Molecular Diagnosis of *Ornethobilharzia Turkestanicum* in Maysan Province - Iraq

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Abstract : *Ornithobilharzia turkestanicum* is one of the Schistosomatidae family that causing cercarial dermatitis in humans, heavy infestations and considerable economic casualties in production of animals like; sheep, goats and cow. The present study were designed to detection of *O. turkestanicum* in sheep at the molecular level by using 28S rRNA gene. Worms were collected from Maysan province/ Southern Iraq. Adult worms were observed in the mesenteric veins of infected sheep and identified as *O. turkestanicum* by traditional methods. Adult flukes used to extraction of DNA from tissue and applied on conventional PCR program to amplification of DNA. Size of PCR product were analyzed by 1% gel agarose was 1009bp in matching with specific DNA ladder (10000bp). Confirmation of molecular diagnosis of *O. turkestanicum* by using sequencing technique. The sequencing of the amplified PCR products were 100% identical between the adult blood flukes and Gene Bank. The conclusion is the molecular techniques are more accurate to detection of adult *O. turkestanicum* in compares with ordinary methods.

Key words: *Ornithobilharzia turkestanicum*, Schistosomatidae, molecular identification, PCR, sequencing.

Introduction

Molecular diagnosis particularly based on PCR-techniques, are within the most important developments that have occurred in diagnostic techniques during studies (1, 2). The molecular methods used for Schistosoma differentiation dates back to 1984, when McCutchan recorded the benefit of rRNA probes for the analysis of restricted fragment length polymorphisms (RFLPs) (3). The molecular technique have been successfully used to determine the sex of the cercaria, the sequencing and cloning of specific genes. Also in deciding on the genetic change and demographic composition of Schistosoma species and strains in the development and the uses of new techniques to generate expressed sequence tags (4).

Detection of specific DNA of the parasite is a selection which is used with many infections like malaria (5), and it presents a chance with *Schistosoma haematobuim* and *S. mansoni* (6, 7). This is also includes *S. haematobuim*, it was estimated using potential designing and was shown to revealing of specific fragments of DNA in adults worm whether when eggs were present in urine, and in 10% of cases the

eggs were not present (8). The detection of sequences of specific fragment of DNA by PCR has proven very useful for the analysis of genetic disease and the detection of miscellaneous infectious disease (9, 10).

Previously, the studies of Orientobilharziasis have been concen-trate on the life cycle, treatment, epidemiology, morphology, diagnosis and prevention (11, 12). However, small amount has been done in the molecular phylogenetic and classific-ation studies of *Orientobilharzia spp.* (13, 14).

The studies on the molecular genetics of *O. turkestanicum* referred to Asiatic strain, with study of genomes of mitochondria for both Chinese and Iranian strain recording present of high levels of symmetry with some local and host particular haplotypes (15, 16). The microscopic examination recognized as the most useful technique used to detect the larval stages of trematodes in the intermediate host (snails). However, microscopic examination technique has low specificity and/or sensitivity in order to the decrease the ability of detection and differentiation of the larval stages of trematodes (17). Therefore, this study were designed to detection of blood worm *O. turkestanicum* at molecular level by using conventional PCR techniques and sequencing method to confirm diagnosis.

MATERIALS AND METHODS

I- Isolation of Adult Worms

The adult flukes of *O. Turkestan-icum* were isolated and collected from the sheep, which possessed from a local slaughterhouse in Maysan province for 6 months periods (from July to the end of December 2015). The total number of isolated worms from infected sheep was (663) divided into (338) male and (325) female. Adults of *O. turkestanicum* were collected from naturally infected sheep from the mesenteric veins were the adult flukes observed. The detection of the adult flukes by using special explanation device were designed to simplify notice of adult flukes inside the mesenteric veins of the sheep.

II- Molecular Methods

A- DNA Extraction

Two hundred fifty specimen are fixed in 70% of ethanol for DNA extraction, the extractions is done by using ZYMO Quick-gDNA™ Mini Prep extraction kit to extract DNA from tissue of worm's body according to manufacturer's recommendation.

B- PCR Detection

Two specific primers (Table 1) were used to amplify a fragment a 1009 (bp) PCR fragment spanning the 28S rRNA gene of *O. turkestanicum* (24).

Table (1): Primers used for amplification of *Ornithobilharzia turkestanicum* 28S rRNAgene.

No.	Name of primer	Sequence	Tm.	M.W
1	O.t-f	5’-CCTTAGTAACTGCGAGTGAACAGG-3’	57.2 °C	7401.9
2	O.t-r	5’-GAGCAAGACAGCAGGATCTCACC-3’	59.6 °C	7051.6

M.W: Molecular Weight

By using iNtRON i-D Maxime™ PCR PreMix (for diagnostic study), 20 ul, pre-aliquoted 96 tubes, which containing 5µl from master mix, 2 µl of DNA samples was added with 1 µl from each primer and 16 µl from DNase/RNase-free distilled water (per each PCR tube), by using thermocycler, the PCR procedure is done by following programs (Table 2): 94 ◦C for 5 minutes initial denaturation followed by 35 cycles of 94 ◦C for 60 seconds for denaturation, 61 ◦C for 80 seconds annealing, and 72 ◦C for 80 seconds extension, 72 ◦C for 10 minutes final extension then holding at 4 ◦C for 5 minutes [24]. Five µl PCR products were analyzed in gel electrophoresis 1% agarose containing 5µl red save stain and illustrated by using ultraviolet illustrating device (Figure. 2).

Table (2): PCR program for amplification of *Ornithobilharzia turkestanicum* gene.

Stages	Steps	Temperature	Time	No. of cycle
Initial denaturation	-	94 ˚C	5 min.	1
Denaturation	1	94 ˚C	60 sec.	35
Annealing	2	61 ˚C	80 sec.	
Extension	3	72 ˚C	80 sec.	
Final extension	-	72 ˚C	10 min.	1
Holding	-	4 ◦C	10 min.	-

C- Sequencing

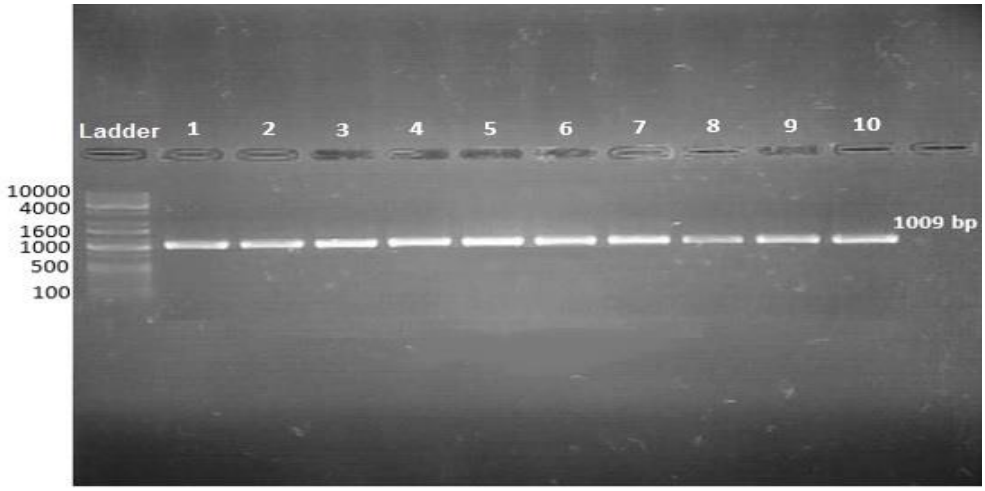

Figure (1): 1% Agarose gel electrophoresis of PCR products at 150v for 30 minutes, and visualized by UV transilluminator. Lane ladder: 10000bp, 1-10 PCR products with 1009bp.

The result of PCR product (longed 1009 pb) analyzed by using 1% gel electrophoresis applied with 10000bp DNA ladder (Fig. 2), all samples were given positive results. PCR product was purified for sequencing by using EZ-10 spin column DNA cleanup minipreps Kit (Bio Basic Inc., Canada). Five PCR products of the *O. turkestanicum* and primers were sending to Macrogen Company, (USA) for sequencing. Homology search was behave using

Basic Local Alignment Search Tool (BLAST) program, which is present at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and Bio Edit program. Then the results were matched with data gained from Gene Bank published ExPASY program which is present at the NCBI online.

Results and Discussions

To detection of *O. turkestanicum* in sheep required macro-examination of the mesenteric veins of sheep, after examination, the adult blood fluke were observed inside mesenteric veins (Fig.2). Microscopic examination, taining and other diagnostic methods are not specific identification of *O. turkestanicum* fluke as compared with molecular methods.

In this study the PCR technique were used to amplification of 28S rRNA gene of *O. turkestanicum* fragment which longed 1009 pb.

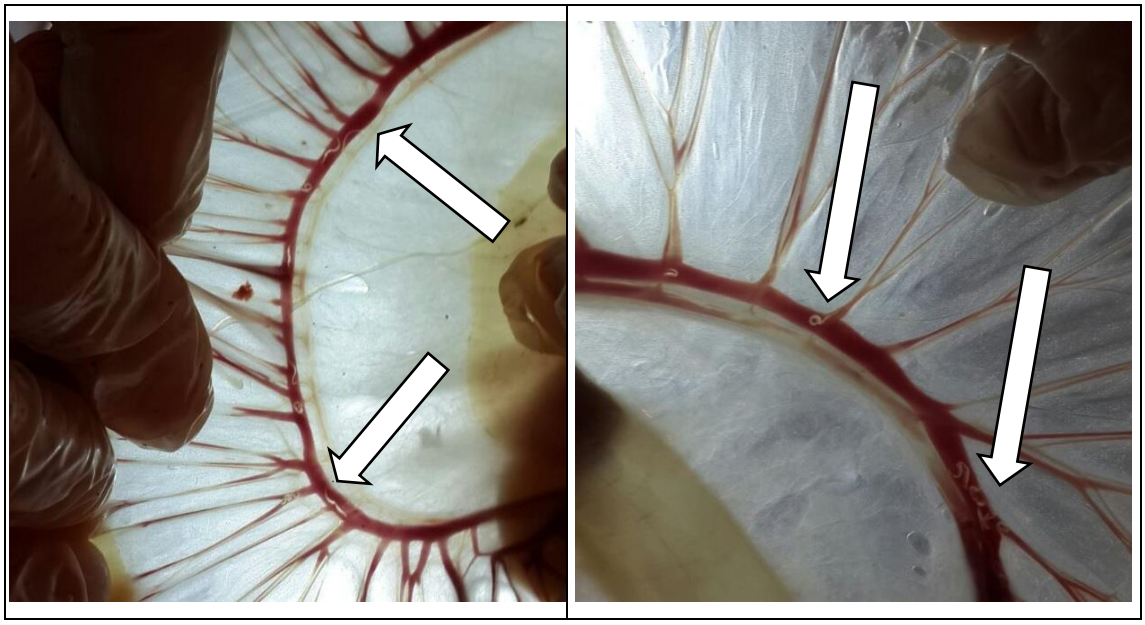

Figure (2): Adult *Ornithobilharzia turkestanicum* inside mesenteric veins of infected sheep.

Sequencing of the PCR products given a 1009 bp long sequences of nucleotide, which were 100% matching between Gene Bank and adult blood flukes. The sequence of the isolated *O. turkestanica* were compared with the characterizing of Hungarian isolate of the single sequence were submitted to Gen Bank under the accession number of EU702749, showed the homology searches of the Gen Bank revealed that the resulting sequence was 100% matching with the *O. turkestanica* 28S rRNA gene of three Chinese and one Iranian isolate (EU436661, EU436660, AJ313461 and AY157254).

O. turkestanicum isolate Gemenc 28S ribosomal RNA gene, partial sequence. Because all samples are matching with the Gene Bank, we take one sample only (No. 1) to decrease the coast of research.

Sequence ID: gb|EU702749.1|Length: 960Number of Matches: 1 Related. Information.

Range 1: 24 to 952 Gen Bank Graphics.

Table (3): Sequences production significant alignments for *O. turkestanicum.*

Alignment statistics for match #1				
Score	**Expect**	**Identities**	**Gaps**	**Strand**
1716 bits(929)	0.0	929/929(100%)	0/929(0%)	Plus/Plus

```
Query  1    GTTGTTTGATCGTGAGGCAATGTGGTGTTTAGGTTGGCTTCTGGCATTACTGCTCTTCCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24   GTTGTTTGATCGTGAGGCAATGTGGTGTTTAGGTTGGCTTCTGGCATTACTGCTCTTCCC  83

Query  61   CAAGTCCAGCAATGAGTACGGCTTCCCATTCTGGCCCATAGAGGGTGAAAGGCCCGTGGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  84   CAAGTCCAGCAATGAGTACGGCTTCCCATTCTGGCCCATAGAGGGTGAAAGGCCCGTGGG  143

Query  121  GGTAGAGACCAAGTGTGACAGTTCTGCTCAGAGCTCCCCTTAGAGTCGGGTTGTTTGTGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  144  GGTAGAGACCAAGTGTGACAGTTCTGCTCAGAGCTCCCCTTAGAGTCGGGTTGTTTGTGA  203

Query  181  ATGCAGCCCAAAGTGGGTGGTAGACTCCATCCAAGGCTAAATACTTACACGAGTCCGATA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204  ATGCAGCCCAAAGTGGGTGGTAGACTCCATCCAAGGCTAAATACTTACACGAGTCCGATA  263

Query  241  GCAAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAAGAGAGAGTAAACAGTGCGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  GCAAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAAGAGAGAGTAAACAGTGCGT  323

Query  301  GAAACCGCTCAAAGGTAAACGGGTGGAGTTGAACTGCAAGCCCTGGGAATTCAGCTGATG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  GAAACCGCTCAAAGGTAAACGGGTGGAGTTGAACTGCAAGCCCTGGGAATTCAGCTGATG  383

Query  361  AGTGTGATTTGTGCTTGGGCATACTGGCCGCCTTCAGTGTCCGTTTAACCGCGGGTGCCT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  AGTGTGATTTGTGCTTGGGCATACTGGCCGCCTTCAGTGTCCGTTTAACCGCGGGTGCCT  443

Query  421  GCCTTATCGGTGGGTGTGTGTGAATCGTTTGCAAGCGACGCACGCACTGTTGGTGTGAGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  GCCTTATCGGTGGGTGTGTGTGAATCGTTTGCAAGCGACGCACGCACTGTTGGTGTGAGG  503

Query  481  GTCTGCTTGTCAGTGCACTTTCTCAGGGTGTTCACCACGACCGGCGCTGCTGCCTGTCTG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  504  GTCTGCTTGTCAGTGCACTTTCTCAGGGTGTTCACCACGACCGGCGCTGCTGCCTGTCTG  563

Query  541  CTATGATCAAACTGGTCGAGTCTGGCATTGTTGGGCTTGGCTGGGTCGGCAGGTGACTCT  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  564  CTATGATCAAACTGGTCGAGTCTGGCATTGTTGGGCTTGGCTGGGTCGGCAGGTGACTCT  623

Query  601  TTGGCTCATTCGTGGGCCGTGAGTGTTACTAGCTGGTTGTAGTGGATCTGTGCGGTGTGT  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  624  TTGGCTCATTCGTGGGCCGTGAGTGTTACTAGCTGGTTGTAGTGGATCTGTGCGGTGTGT  683

Query  661  CGGAGATGGCGGCTTCGCATGCGTGCTTAGACCTGCGGACATTGTTGAGTCTGGTCGGTT  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  684  CGGAGATGGCGGCTTCGCATGCGTGCTTAGACCTGCGGACATTGTTGAGTCTGGTCGGTT  743

Query  721  TGTTACTGGCTTCGGCTGGTCGGCTGATGGCTTGATTTTGTTGCGTTGGCAGTTGCGTGT  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  744  TGTTACTGGCTTCGGCTGGTCGGCTGATGGCTTGATTTTGTTGCGTTGGCAGTTGCGTGT  803

Query  781  GTGAACGGCTTGGGCCCATAGTCTGTGGTGTAGTGGTAGACGATCCACCTGACCCGTCTT  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  804  GTGAACGGCTTGGGCCCATAGTCTGTGGTGTAGTGGTAGACGATCCACCTGACCCGTCTT  863

Query  841  GAAACACGGACCAAGGAGTTTAACATGTGCGCGAGTCATTGGGTGTTACGAAACCCAAAG  900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  864  GAAACACGGACCAAGGAGTTTAACATGTGCGCGAGTCATTGGGTGTTACGAAACCCAAAG  923

Query  901  GCGAAGTGAAGGTAAAGGTTCGGCTTGTC  929
            |||||||||||||||||||||||||||||
Sbjct  924  GCGAAGTGAAGGTAAAGGTTCGGCTTGTC  952
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Figure (3): Sequencing of *Orientobilharzia turkestanicum* isolate Genomic 28S ribosomal RNA gene as compared with standard data from Gene Bank.

The studies of *O. turkestanicum* are limited in Iraq. The first detection of intermediate host for *O. turkestanicum* in Iraq is recorded after characterization of the snail host that was the snail *Lymnaea teneraeuphratica Mousson*, and was found to be naturally infected with apharyngeal brevifurcate furcocercous cercariae (18). After that, many studies were used that depending on DNA extracted from *O. turkestanicum* adult worms (19, 20) or larval stages include cercaria (21, 22) to diagnosis and differentiation of *O. turkestanicum* from other types of Schistosoma. Some studies were used nested PCR (21), and others used real-time quantitative PCR (19) but the most of these studies were used conventional PCR (20, 23).

Some of these studies used 28S ribosomal RNA (rRNA) gene (23, 24) and others used MicroRNA (miRNA) gene (19). This study was designed to used 28S rRNA gene extracted from tissue of adult *O. turkestanicum* males and females.

Many of studies are done in Iraq to study of *O. turkestanicum*, these studies are either be designed to found the pathogenesis of *O. turkestanicum* in the final host (25), or to study the intermediate host of this worms (11).

There are many methods used to diagnosis of *O. turkestanicum* in infected animals, detection of parasite egg in the feces of infected animals on life, but it recorded when the infection is in chronic phase (21). Another method is separating of adult flukes from mesenteric or portal veins from slaughtered animals and staining to observation the internal organs of *O. turkestanicum* to differentiate from other types of Schistosoma, or examination of the intermediate host (snails) for detection of cercaria that may take several weeks in laboratory. These methods are useful but inaccurate, time consuming and take intensive effort to the worker (21).

The molecular techniques applications given an accurate assessment of the infection that will occur by specific organisms and detection of infections that could not be detected by the traditional methods, especially detection of larval stages of trematodes (22).

On the other hand the sequencing of *O. turkestanicum* to confirm the Iraqi strain is matched with Gene Bank and showed 100% identical with *O. turkestanicum* 28S rRNA gene which recorded as accession number (EU702749) with the length (960). There is no differed from Chinese and Iranian isolate. But some studies detection of *O. turkestanicum* in Hungarian red deer by sequence recorded there is a difference in single nucleotide from other Iranian and Chinese isolate (EU436659, AF167092) and difference in two nucleotides from one Chinese isolate (EU436662) and recorded that these sequences from Iran and China are the only *O. turkestanicum* sequences available in the Gen Bank database(24).We concluded that the molecular techniques (PCR and sequencing are more accurate to detection of adult *O. turkestanicum* infected sheep rather than traditional methods and the type and strain that found in Iraq was the same that in Iran and China which mean have the same intermediate host in these regions.

References

1. Weiss, J. B. (1995). DNA probes and PCR for diagnosis of parasitic infections. Clin. Microbiol. Rev., 8 (1):113-30.
2. Bell, A. and Ranford-Cartwright, L. (2002). Real-time quantitative PCR in Parasitology. Trends Parasitol., 1; 18 (8): 337-42.

3. McCutchan, T. F.; Simpson, A. J.; Mullins, J. A.; Sher, A.; Nash, T. E.; Lewis, F. and Richards, C. (1984). Differentiation of schistosomes by species, strain, and sex by using cloned DNA markers.. Proc Natl Acad Sci USA, 81 (3): 889-93.
4. Pontes, L. A.; Dias-Neto, E. and Rabello, A. (2002). Detection by polymerase chain reaction of *Schistosoma mansoni* DNA in human serum and feces. Am. J. Trop. Med. Hyg., (66): 157–162.
5. Mharakurwa, S.; Simoloka, C.; Thuma, P. E.; Shiff, C. J. and Sullivan, D. J. (2006). PCR detection of *Plasmodium falciparum* in human urine and saliva samples, Malaria J., (5): 103.
6. Enk, M. J.; Oliveira, G.; Silva, E. and Rodrigues, N. B. (2010). A salting out and resin procedure for extracting *Schistosoma mansoni* DNA from human urine samples, BMC Research Notes, (3): 115.
7. Ibironke, O. A.; Phillips, A. E.; Garba, A.; Lamine, S.M. and Shiff, C. J. (2011). Diagnosis of *Schistosoma haematobuim* by detection of specific DNA fragments from filtered urine samples, Ame. J. of Trop. Med. and Hyg., 84 (6): 998–1001.
8. Ibironke, O. A.; Koukounari, A.; Asaolu, S.; Moustaki, I. and Shiff, C. J. (2012). Validation of a new test for *Schistosoma haematobium* based on detection of the Dra1 DNA repeat fragment in urine: evaluation through latent class analysis, PLoS Neglected Tropical Diseases, 6 (1): 1464.
9. Lucena, W. A.; Dhalia, R.; Abath, F. G. C.; Nicolas, L.; Regis, L. N. and Furtado, A. F. (1998). Diagnosis of *Wuchereria Bancrofti infection* by the polymerase chain reaction using urine and day blood samples from amicro filaraemic patients. Trans R. Soc. Trop Med Hyg, (92): 290-293.
10. Rodrigues, E. H. G.; Brito, M. E. F.; Mendonça, M. G.; Werkhäuser, R. P.; Coutinho, E. M.; Souza, W. V.; Albuquerque, M. F. P. M.; Jardim, M. L. and Abath, F. G. C. (2002). Evaluation of PCR for Diagnosis of American Cutaneous Leishmaniasis in an Area of Endemicity in Northeastern Brazil. J Clin. Microbiol., (40): 3572-3576.
11. Al-To'mma, M. A. M. (1997). A study of some pathological effects of *Orientobilharzia turkestanicum* on fresh water snail *Lymnaea auricularia*, M.Sc. thesis, University of Basrah, pp. 1-51.
12. Wang, C. R.; Xu, L. M.; Wang, H. X.; Qiu, J. H.; Xu, M. Q.; He, G. S.; Jiang, Y.; Yu, W. C. and Wang, Q. F. (2000). Application of dot-immuno gold filtration assay in detection of Orientobilharziasis of sheep. Heilongjiang J. Ani. Sci. Vet. Med., (5):24–25.
13. Attwood, S. W.; Upatham, E. S.; Meng, X. H.; Qiu, D. C. and Southgate, V. R. (2002). The phylogeography of Asian Schistosoma (Trematoda: Schistosomatidae). Parasitology, (125): 99-112.
14. Sara, V. B. and Loker, E. S. (2005). Can specialized pathogens colonize distantly related hosts? Schistosome evolution as a case study. PLoS Pathog, 1 (3):e38.
15. Li, L.; Yu, L. Y.; Zhu, X. Q.; Wang, C. R.; Zhai, Y. Q. and Zhao, J. P. (2008). *Orientobilharzia turkestanicum* grouped within African schistosomes based on phylogenetic analyses using sequences of mitochondrial genes. Parasitol. Res., (102): 939– 943.
16. Webster, B. L. and Littlewood, D. T. J. (2012). Mitochondrial gene order change in Schistosoma (Platyhelminthes: Digenea: Schistosomatidae). Int. J. Parasitol., (42): 313–321.
17. Kaplan, R. M.; Dame, J. B.; Reddy, G. R. and Courtney, C. H. (1997). The prevalence of Fasciola hepatica in its snail intermediate host determined by DNA probe assay. Int J. Parasitol, (27): 1585–93.
18. Machattie, C. (1936). A preliminary note on the life history of *Schistosoma turkestanicum* (Skerjabin, 1913), Transactions of the Royal Society of Tropical Medicine and Hygiene, 30 (1): 115-128.
19. Wang, C. R.; Xu, M. J.; Fu, J. H.; Nisbet, A. J. and Chang, Q. C., *et al.* (2012). Characterization of MicroRNAs from *Orientobilharzia turkestanicum*, a Neglected Blood Fluke of Human and Animal Health Significance. PLoS ONE, 7 (10): e47001.
20. Lawton, S.P. and Majoros, G. (2013). A foreign invader or a reclusive native? DNA bar coding reveals a distinct European lineage of the zoonotic parasite *Schistosoma turkestanicum* (syn. *Orientobilharzia turkestanicum* (Dutt and Srivastava, 1955)), Infection, Genetics and Evolution, (14): 186–193.
21. Motamedi, G. R.; Ghorashi, S. A.; Paykari, H.; Dalimi, A. H.; Salehi Tabar, R.;

Motamedi, N. and Karimi, Gh. R. (2008). Detection of *Ornithobilharzia turkestanicum* cercaria (trematoda) by nested-PCR in intermediate host snail, *Lymnaea gedrosiana*. Arch Razi Inst., (63): 35– 40.

22. Yakhchali, M.; Mirrajei, S. Y. and Malekzadeh-Viayeh, R. (2013). Detection of Infection with Larval Stages of *Ornithobilharzia turkestanicum* using PCR in Field-Collected Snails of *Lymnaea gedrosiana* from Northwestern Iran. Iranian J. Parasitol., 8 (4): 627-633.
23. Wang, C. R.; Li, L. and Ni, H. B. (2009). *Orientobilharzia turkestanicum* is a member of Schistosoma genus based on phylogenetic analysis using ribosomal DNA sequences. Exp. Parasitol., 121 (2): 193–7.
24. Majorosa, G.; Dan, A. and Erdelyi, K. (2010): A natural focus of the blood fluke *Orientobilharzia turkestanica* (Skrjabin, 1913) (Trematoda: Schistosomatidae) in red deer (*Cervus elaphus*) in Hungary. Vet. Par. Int. Sci. j., (170): 218–223.
25. Al-To'mma, Z. A. M. (2011). The liver cirrhosis caused by *Orientobilharzia turkestanicum* in Basra governorate, J. of College of Education, Thi-Qar, 5 (1): 81-87.