



# Evaluation of Semi-Nested PCR Compared with Indirect-ELISA to Diagnose Human Fasciolosis

Gita Alizadeh<sup>1</sup>, Mojgan Aryaeipour<sup>1</sup>, Mehdi Mohebali<sup>1,2</sup>, Gholam Reza Mowlavi<sup>1</sup>, Vahid Raissi<sup>1</sup>, \*Mohammad Bagher Rokni<sup>1,2</sup>

1. Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2. Center for Research of Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran

\*Corresponding Author: Email: roknimoh@tums.ac.ir

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## Abstract

**Background:** We aimed to compare semi-nested PCR with indirect ELISA to diagnose human fasciolosis.

**Methods:** Overall, 70 serum samples were collected from different areas in Iran suspected for fasciolosis. Individuals were classified based on diagnostic of fasciolosis and habitat in endemic areas. Finally, all serum samples were tested by indirect ELISA (using secretory excretory antigen) and semi-nested PCR (using ITS1 gene). The study was conducted in the School of Public Health, Tehran University of Medical Sciences, Iran in 2021.

**Results:** Significant differences were found between agreement and similarity of patients' results of indirect ELISA and semi-nested PCR 94.46% and 98.4% respectively (Cohen's kappa  $\geq 0.6$ ;  $P \leq 0.05$ ). No cross-reactions were observed with other parasitic diseases (toxocariasis, hydatidosis, strongyloidiasis, toxoplasmosis, cutaneous leishmaniasis, taeniasis and trichinosis). 69.84% of samples were positive by both techniques. In addition, the percentage of agreement and similarity between the results of the two techniques based on habitat in endemic areas was 88.9-100% and 97.7-100%, respectively (Cohen's kappa  $\geq 0.6$ ;  $P \leq 0.05$ ).

**Conclusion:** Semi-nested PCR could be a suitable method for following up on patients' treatment and a confirmatory method for ELISA as for diagnosis of human fasciolosis.

**Keywords:** Human fasciolosis; Semi-nested polymerase chain reaction; Parasitology

## Introduction

Human fasciolosis caused by *Fasciola spp.* is considered as an important parasitic disease not only for human but for ruminants as well (1,2). It is not usually prevalent in developed countries but has been reported from many countries in the world including Iran (3). It is an important parasitic disease and causes health, veterinary and economic problems in many countries in the world such as Iran. Humans and ruminants are

the definitive hosts and *Lymnaea spp.* are the intermediate hosts (4, 5).

People are infected usually via ingesting metacercariae of the parasite through water, vegetables, and local foods (5, 6). Clinical symptoms include liver involvement, fever, nausea, anemia, and gastrointestinal disorders (4, 7, 8).

For diagnosis of fasciolosis, although parasitological methods are the golden standard methods, but due to diurnal alternation in egg excretion,



low sensitivity of stool exam, and need the special techniques, researchers prefer to use of serological tests including ELISA (4,6,9). The problem of serological tests is that it is impossible to detect the disease after treatment and it needs to some molecular tests (10-12). Recent molecular studies on urine and feces using the nested PCR method have shown promising results in this regard (13, 14).

To verify the difference between molecular and serological methods on diagnosis of human fasciolosis we compared both semi-nested PCR and indirect ELISA for the first time in Iran.

## Materials and Methods

### Ethics statement

All tests on human serum samples were performed on individuals according to the instructions of the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran with approval number of IR.TUMS.SPH.REC.1399.327.

### Subjects

The present study was performed on 70 human serum samples described in table 1. It was conducted in the Helminthology Laboratory, Department of Parasitology and Mycology, School of Health, Tehran University of Medical Sciences, Tehran, Iran from 2020 to 2021.

**Table1:** Description of human serum samples in different groups

<i>Classification of serum samples</i>	<i>Number of serum samples</i>	<i>Group</i>
Suspicious samples for <i>Fasciola</i> spp. (Untreated)	34	A
Positive stool and serum samples for <i>Fasciola</i> spp.	10	<sup>1</sup> B
Negative stool and serum samples for <i>Fasciola</i> spp.	12	<sup>2</sup> C
Positive for other parasitic diseases by indirect ELISA (toxocariasis, hydatidosis, strongyloidiasis, toxoplasmosis and cutaneous leishmaniasis)	7	D
Positive serum samples for <i>Fasciola</i> spp. by indirect ELISA (treated)	7	E
<b>Total serum samples</b>	<b>70</b>	

<sup>1</sup> Positive control

<sup>2</sup> Negative control

### Antigen preparation and Serological method (indirect ELISA)

Preparation and protein concentration of the ES antigen from adult *F. gigantica* was performed as reported already (7). All serum samples were examined by indirect ELISA method based on a previous study (13).

### Molecular method

After DNA extraction of serum samples, PCR and semi-nested PCR were performed.

### DNA extraction from serum

We extracted DNA from serum using the genomic DNA kit (DNG™-PLUS) (14) and AddPrep, both of which were suitable for extrac-

tion of genomic DNA from fascioliasis. DNA extraction based on AddPrep Genomic DNA Kit (Product Code: 10023; Korea) (13).

### Primer's design

The primers were synthesized by Bioneer Company (Korea) (13) and deposited in GenBank. Table 2 shows the sequence of primers used.

### PCR and semi-nested PCR assays

A sequence of 700 bps and 500 bps in the ITS1 were amplified by PCR and semi-nested PCR, respectively. Table 3 shows the temperature specifications for PCR and semi-nested PCR amplification cycles. Finally, PCR products were divided into 1.5% agarose gel with Simply Safe (Fig. 1).

**Table 2:** Sequences of primers used for PCR and semi-nested PCR amplification

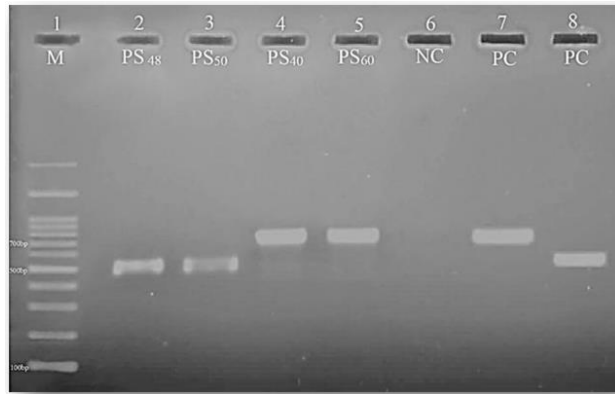
<i>Primers</i>	<i>Sequences</i>	<i>References</i>
ITS1 (Forward) <sup>1,2</sup>	TTGGCTGCGCTCTTCATCGAC	(15)
ITS1 (Reverse) <sup>1</sup>	TTGCGCTGATTACGTCCCTG	(15)
ITS1 (Reverse) <sup>2</sup>	CGACGTACGTGCAGTCCA	(16)

<sup>1</sup>PCR

<sup>2</sup>semi-nested PCR

**Table 3:** Temperature specifications for PCR and semi-nested PCR amplification cycles

<i>Assay</i>	<i>Initiation (1 cycle, 5min)</i>	<i>Denaturation (35 cycles, 30 sec)</i>	<i>Annealing (35 cycles, 30 sec)</i>	<i>Extension (35 cycles, 1 min) Final extension (1 cycle, 5 min)</i>
PCR	95 °C	95 °C	55 °C	72 °C
semi-nested PCR	95 °C	95 °C	58 °C	72 °C



**Fig. 1:** Semi-nested PCR (500 bp) and PCR (700 bp) patterns of HF by ITS1 region *Fasciola* spp. Lane 1(M): 100bp DNA ladder, Lane 2 and 3: positive serum samples by semi-nested PCR, Lane 4 and 5: positive serum samples by PCR, Lane 6: Negative control, Lane 7 and 8: Positive control

**Sequencing of ITS1 region of samples**

Candidate-positive samples were purified and sequenced by Niagen noor company. In addition, sequencing sources were examined using chro-

mas software and the nucleotides were extracted with the Fasta extension and registered in the gene bank. (Table 4).

**Table 4:** Gene Registration results

<i>Isolate code</i>	<i>Species</i>	<i>Host</i>	<i>Sequence analysis</i>	<i>ITS 1 types</i>	<i>Accession no.</i>	<i>Semi-nested PCR analysis</i>
H1	<i>F. gigantica</i>	Human	<i>F. gigantica</i>		OK136247	<i>F. gigantica</i>
H2	<i>F. gigantica</i>	Human	<i>F. gigantica</i>		OK136248	<i>F. gigantica</i>

### Statistical analysis

Statistical analysis was done using SPSS version 26 (IBM Corp., Armonk, NY, USA). Cohen's kappa was used for data analyzing.

### Results

The results of this study are classified and presented based on four sections by tables 5-7, and fig. 1 and 2. Semi-nested PCR and PCR patterns

of fasciolosis by ITS1 of *Fasciola* spp. Are shown in Fig. 1. Comparison of the diagnosis of fasciolosis based on indirect ELISA and semi-nested PCR is reported in table 5. Compliance of semi-nested PCR and indirect ELISA on all serum samples of fasciolosis in terms of habitat in endemic areas is presented in table 6. Finally, results of clinical signs and phylogenetic tree are depicted in Fig. 2.

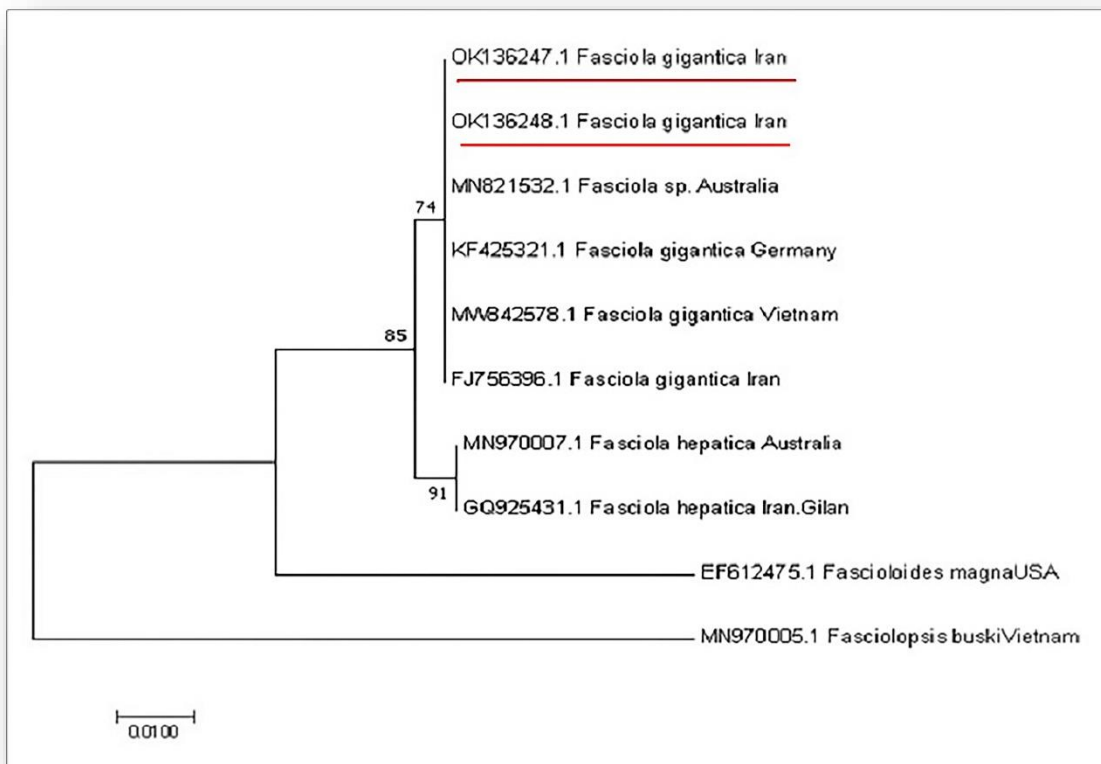


Fig. 2: Phylogenetic tree

Table 5 shows that there was a high similarity on the patients' results (over 98%), and only less than 4% difference in results by two methods (Coefficient cohen's kappa: 0.96;  $P=0.02$ ). Accordingly, the percentage similarity of patients' results and the percentage of agreement (based on Coefficient cohen's kappa) were detected at 98.4% and 94.46%, respectively.

According to table 6, similarity and agreement between indirect ELISA and semi-nested PCR results was observed in the diagnosis of fasciolosis in individuals based on habitat in endemic areas of fascioliasis. In patients' cases, agreement was reached and the range of percentage similarity and agreement in 2 group of individuals was reported 98-100% and 89-100%, respectively (Coefficient cohen's kappa  $\geq 0.6$ ;  $P=0.01$ ).

**Table 5:** Similarity and agreement of results of indirect ELISA and semi-nested PCR in all samples for fasciolosis

Samples N (%)	Semi-nested PCR	ELISA (%)		Coefficient cohen's kappa	Agreement (%)	P- value
		Negative	Positive			
Group A (Untreated) 34(54)	Negative	0(0.0)	0 (0.0)	1	100	0.01
	Positive	0 (0.0)	34 (100)			
Group B (Un- treated) 10(15.9)	Negative	0(0.0)	0 (0.0)	1	100	0.01
	Positive	0 (0.0)	10 (100)			
Group C 12(19)	Negative	11(91.67)	0 (0.0)	0.88	80.66	0.03
	Positive	1 (8.33)	0 (0.0)			
Group D 7(11.1)	Negative	7(100)	0 (0.0)	1	100	0.01
	Positive	0 (0.0)	0 (0.0)			
Total 63(100)	Negative	18 (28.5)	0 (0.0)	0.96	94.46	0.02
	Positive	1(1.6)	44(69.9)			
Group E (Treated) 7(10)	Negative	0(0.0)	7(100)	0.0	0.0	0.97
	Positive	0 (0.0)	0 (0.0)			
Total 70(100)	Negative	18(25.7)	7 (10)	0.95	82.3	0.02
	Positive	1 (1.45)	44 (62.85)			

**Table 6:** Similarity and agreement of results of indirect ELISA and semi-nested PCR based on habitat in endemic areas for the diagnosis of fasciolosis

Habitat in endemic areas N (%)	Semi-nested PCR	ELISA(%)		Coefficient cohen's kap- pa	Agreement (%)	P- value
		Negative	Positive			
Yes 42(100)	Negative	0(0.0)	0 (0.0)	0.91	88.9	0.02
	Positive	1 (2.3)	41 (97.7)			
No 21(100)	Negative	18(85.72)	0 (0.0)	1	100	0.01
	Positive	0 (0.0)	3 (14.28)			

The phylogenetic tree ITS1 showed that two human samples were *F. gigantica*, which indicates

that *F. gigantica* genotype can infect humans in endemic area of Iran (Fig. 2 and Table 7).

**Table 7:** Gene registration result in this study and comparing to other gene registrations

Isolate code	Species	Host	ITS1 types Acces- sion no.	Sequence analysis
H1	<i>F. gigantica</i>	Human	OK136247	<i>F. gigantica</i>
H2	<i>F. gigantica</i>	Human	OK136248	<i>F. gigantica</i>
H3	<i>F. spp.</i>	Cattle	MN821532	<i>F. spp.</i>
H4	<i>F. gigantica</i>	P. clumella	KF425321	<i>F. gigantica</i>
H5	<i>F. gigantica</i>	Bovin	MW842578	<i>F. gigantica</i>
H6	<i>F. gigantica</i>	Cattle	FJ56396	<i>F. gigantica</i>
H7	<i>F. hepatica</i>	Pig	MN970007	<i>F. hepatica</i>
H7	<i>F. hepatica</i>	Human	GQ925431	<i>F. hepatica</i>
H8	<i>F. magna</i>	deer	EF612475	<i>F. magna</i>
H9	<i>F. buski</i>	Pig	MN970005	<i>F. buski</i>

## Discussion

The present study was designed to detect the agreement between indirect ELISA and semi-nested PCR techniques in the diagnosis of fasciolosis and in terms of habitat in endemic areas.

Fasciolosis, caused by *F. hepatica* (worldwide) or *F. gigantica* (Africa and Asia), is a dangerous parasitic disease, introduced by the WHO (17, 18). Fasciolosis, in the asymptomatic, acute, and chronic forms is associated with short-term and long-term effect on human health (19). Infection of fasciolosis causes biliary tract inflammation and obstruction (20). In Iran, although epidemics of fasciolosis in past have been occurred in the northern region, due to the changing epidemiological pattern and several reports of fasciolosis in another parts of Iran in recent years, the possibility of new focus of fasciolosis will not be denied in the future (21).

In our study, individuals based on habitat in endemic areas and people whose residence was in non-endemic areas were examined. The obtained serological and molecular results show the importance and attention to endemic regions of Iran to prevent the increasing process of fascioliasis and control this disease. Moreover, although there are various methods used to the diagnosis of fasciolosis, each has its limitations and disadvantages. For example, the presence of cross-reactions in serological diagnosis, ineffectiveness of parasitological methods in the diagnosis of acute and chronic stages of fasciolosis are reported (7,8-12,13), whereas molecular methods are more specific (Real-time PCR or semi-nested PCR) than other methods (22-25). Similarly, comparisons between diagnostic methods (molecular, serological and microscopic) have always been proposed in parasitic diseases to reach a preferred method.

There was more than 94% agreement and about 99% similarity on the results of diagnostic methods in this study. ELISA (use of ESPs) and molecular (DNA-based) methods for diagnosis of clonorchiasis were used, like fascioliasis (26). Besides, copro-ELISA and copro-PCR were able to

detect intestinal capillariasis in 95% and 83% of fecal samples, respectively (27). In another study, 100% similarity of nested PCR (*B1 T. gondii* gene) and ELISA (IgG Ab) results (63% of samples by two methods) was observed in the diagnosis of chronic toxoplasmosis (28). In Wichmann et al study, the use of PCR was very important in the detection of 100% of cell-free parasite DNA (CFPD) of *Schistosoma haematobium* in human plasma samples (29), and in another study by nested PCR (use of partial ribosomal DNA of *Strongyloides stercoralis*), 75% of negative human fecal samples (by agar plate culture) was reported as positive (30).

Some studies have been performed on animal serum samples. One study used pig serum samples for diagnosis of cysticercosis and ocular toxoplasmosis by ELISA and PCR assay. There was 100% similarity in diagnosis of porcine cysticercosis by two methods (32.8% positive, 67.2% negative) (31). The diagnosis of ocular toxoplasmosis by ELISA and nested PCR methods showed 60% and 46% similarity, respectively (32).

Owing to the fact that diagnosis of fasciolosis by various methods is of great importance, in this section, studies on fasciolosis has been discussed. Duplex PCR (use of *cox1* gene) could be a rapid and sensitive method for detection of *Fasciola* spp. (33). There were similarities between the results of semi-nested PCR in present study and other studies for detection of fascioliasis (33-35). Nested PCR was able to detect all cases (as in-group B in the present study) (34), and 100% of samples were detected by ELISA (as in-group A in the present study) (35).

Likewise, PCR and recombinase polymerase amplification (RPA) were used to diagnose of fascioliasis infection. RPA and PCR were able to identify 87% and 66% of negative stool samples by microscopy method. There was no cross-reactivity and the specificity of PCR and RPA was 100% (36). Besides, there was kappa agreement (0.72) among microscopic diagnostic tests, PCR and loop-mediated isothermal amplification (LAMP), based on *IGS* and examine on stool sheep samples, and the LAMP could be used in

the field conditions of fascioliasis in animals (37). The results of another study showed that the subjects who were positive for ELISA, their stool and urine samples were 96% and 90% positive by nested PCR (use of ITS1 such as present study) respectively, and only 20% of samples was diagnosed by parasitology method (13). In addition, using High-Resolution Melting (HRM) there was a powerful, rapid, and sensitive technique for an epidemiological survey and molecular identification between *F. hepatica* and *F. gigantica* (38).

According to the finding of our study, semi-nested PCR could be used to determine the genotyping of the pathogen in humans. Sometimes, we do not have access to the source of the parasite. Because the adult worm is in the liver, and the eggs are excreted alternately, with semi-nested PCR and checking cell-free DNA in the serum of individuals and with obtaining sequences to reach the genotyping of the pathogen, we may reach a final diagnosis. semi-nested PCR can be very useful in diagnosing the disease and its epidemiology.

Our results suggest that molecular techniques can play an effective role in approving or rejecting reference methods (agreement about 95%). This study was able to reveal clearly the difference between agreement and similarity in the results of ELISA and semi-nested PCR by Kappa index. In addition, semi-nested PCR was a suitable method for following-up of patients because 100% of samples treated were negative by semi-nested PCR. ELISA has not been able to diagnose of fasciolosis in-patient with immune deficiency due to the lack of antibodies or the presence of low antibodies in body fluids, so, needed to approval by semi-nested PCR. Further research investigating other molecular techniques, especially real-time PCR, to semi-nested PCR would be a very useful follow-up to this study.

## Conclusion

We described the results, including the agreement between the ELISA and semi-nested PCR, as well as the relationship between this agreement

and settlement in endemic areas. In the same way, a more than 94% agreement was observed between the results of the two methods. In all, we have shown that semi-nested PCR could be suitable method for following up on patients' treatment and a confirmatory method for ELISA in terms of diagnosis of human fasciolosis.

## Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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