



Detection Cell-Free DNA (cfDNA) Using Nested-PCR as a Diagnosis Tool for Human Fascioliasis Infection

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Abstract

Background: We aimed to detect *Fasciola* specific deoxyribonucleic acid (DNA) by nested-PCR assay on human stool and urine samples and compare the results with the respective ELISA diagnostic assay.

Methods: Overall, 206 clinically suspected cases of fascioliasis were enrolled in the study. Blood samples were collected from all the patients, and serum samples were isolated. ELISA assay, using *Fasciola* somatic antigen (SA), was carried out to detect anti *Fasciola* antibodies for the collected sera. DNA was randomly extracted from 25 stool and 10 urine samples of seropositive individuals and was evaluated by conventional PCR and nested PCR methods. The nested-PCR results were confirmed by sequencing the 430 bp region of ribosomal ITS1 gene. Stool and urine samples from patients with different parasitic diseases and 25 stool samples from healthy individuals served as controls. Urine samples were collected from 10 healthy controls as well.

Results: Fascioliasis was detected by ELISA in 24.8% of the individuals. Of these, 25 seropositive patients were randomly assigned to the study. *Fasciola* DNA was identified in the stool samples of 96% of seropositive patients by nested PCR but ova of *Fasciola* was detected by parasitology methods in only 20% of seropositive cases. *Fasciola* DNA was identified in 90% of the urine samples by nested PCR. No cross-reactions were observed with other parasites.

Conclusion: Detection of cfDNA in stool and urine samples has high accuracy and thus can be used for the diagnosis of *Fasciola* infection in human.

Keywords: Fascioliasis; Serodiagnosis; Nested-PCR; Human; Cell-free DNA

Introduction

Fascioliasis is a zoonosis parasitic disease caused by *Fasciola* spp. and is established in most regions of the world. Humans are infected by ingesting of encysted metacercaria associated with aquatic

or semi aquatic plants, or by drinking water contaminated with float metacercaria (1). Fascioliasis is a major health problem especially in north of Iran (2) and sporadic cases have been reported

from other provinces (3). The sequencing of the PCR-amplified 16S rRNA gene has become a common approach to microbial community investigations in the fields of human health and environmental sciences (4). This approach, however, is difficult when the amount of DNA is too low to be amplified by standard PCR (5). Nested PCR can be employed as it can amplify samples with DNA concentration several-fold lower than standard PCR. However, potential biases of nested PCR that could affect measurement of community structure have received little attention (6). The sensitivity of conventional parasitological methods is inadequate for diagnosis of fascioliasis (7). For a definite diagnosis, it is necessary to repeatedly examine stool samples over several consecutive days. The sensitivity of serological methods has been superior respective to the parasitological methods in the diagnosis of fascioliasis (8). Nevertheless, the efficacy of the both approaches has not been 100%. Therefore, there is a need for developing new methods with higher sensitivity and specificity. Detecting molecular biomarkers (i.e. circulated cell-free DNA (cfDNA)) in body fluids (blood,

urine, sputum, stool, etc.) is a novel strategy in biomedical research (9). The detection of cfDNA provides a non-invasive method employing basic nucleic acid amplification procedures for diagnosis of parasitic infections (10).

This study aimed to evaluate *Fasciola* specific deoxyribonucleic acid (DNA) detection by nested-PCR assay in human stool and urine samples and compare the results with ELISA diagnosis assay for fascioliasis.

Materials and Methods

Sample collection

Throughout 2011–2017, 206 blood samples were collected from all the patients' referred to Helminthological Laboratory of the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Patients referred from different parts of the country (Fig. 1).

The protocol was approved by Tehran University of Medical Sciences, Ethics Committee (No code: 31382).



Fig. 1: Distribution of fascioliasis patients in different provinces of Iran

They were referred by physicians from private clinics, or hospitals due to the occurrence of clinical symptoms or eosinophilia in peripheral blood (11).

Informed consent was taken from each patient. A questionnaire including information on location,

diet, vegetable consumption, travelling to northern Iran and clinical symptoms were filled out for each patient. Blood was taken from all patients and sera were stored in refrigerator until examination. All sera were tested using indirect ELISA established already. Patients registered positive

for fascioliasis were identified, among them, any patient whose clinical characteristics and demographic data were completely available was included in this analytical study (11). Overall, 25 fresh stool samples and 10 urine samples were randomly collected from serological positive patients. It also included 25 fresh stool samples and 10 urine samples from healthy individuals and treated fascioliasis patients as negative control. In addition, stool and urine samples were collected from patients with various parasitic diseases including strongyloidiasis, toxocarasis, trichostrongylosis, toxoplasmosis and hydatidosis. A part of stool samples stored in ethanol 70% and others stored in formalin. Urine samples were stored in -20°C until use.

Serological method

The collected serum samples were analyzed using ELISA method (12). Finally, absorbance was measured by an ELISA reader at 492 nm.

Antigen preparation

The livers infected with *Fasciola* spp. were provided from abattoir across the Tehran City and then transferred to the center of Helminthic Diseases in Tehran University of Medical Sciences. After the isolation of parasites, they were rinsed in normal saline and homogenized by PBS (pH 7.2) by an electrical device. The resultant supernatant was stored at 4 °C until use (13).

ELISA

The ELISA protocol was performed as reported previously (13). The somatic antigen with the concentration of 1 mg/ml (100 µl) was poured into 96-well plates and incubated overnight at 37 °C before mixing with gelatin 1% (200 µl). After three episodes of washing with PBS/Twin 20, diluted (1:500) serum samples (100 µl) were poured into antigen-coated wells. The mixture was then incubated in 37 °C for 30 min. Positive and negative controls were run in parallel (i.e. serum samples from *Fasciola* infected and healthy individuals respectively). After washing the mixture with PBS/Twin 20 for 3 times, the wells were incubated with diluted (1:12000) peroxi-

dase-conjugated goat anti-human IgG in 37 °C for 30 min. After another round of washing, 100 µl substrate included OPD (o-phenylene diamine dihydrochloride) was added. Finally, the reaction was ceased by using 50 µl H₂SO₄ solution (12.5%) as the stopper reagent. The absorption was recorded in 492 nm

Parasitological method

All fecal samples were examined using formalin ether concentration technique and microscopic examination.

Molecular method

DNA was extracted from fecal and urine samples then subjected to conventional PCR and nested PCR.

DNA extraction from fecal

A part of each fecal sample was preserved in 70% ethanol at room temperature for DNA isolation. Stool samples were washed twice with phosphate buffered saline (PBS) to remove the ethanol followed by centrifugation at 2000 × g for 5 min. Approximately 1 g stool, diluted in 10 mL PBS, was subjected to 5 cycles of freezing (liquid nitrogen) and thawing (in boiling water) (-170 °C for 5 min and 90 °C for 5 min). Samples were mixed with 200 mg of glass beads (0.45–0.52 mm diameter) and shaken vigorously for 5 min. Total genomic DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

DNA extraction from Urine

A part of each urine sample was frozen for DNA isolation. To remove supernatant, urine samples were centrifuged at 2000×g for 5 min. DNA in sediment was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Primers design

Based on alignment of different sequences related to *Fasciola* species, deposited in GenBank, two

sets of primer pairs were designed for nested PCR using Gene Runner software to amplify a 700 bp target in the first PCR round and a 430 bp target sequence in the second PCR round within the nuclear ribosomal internal transcribed spacer I (ITS1) region.

For the first amplification round, we used the primers as forward (5'- ACCGGTGCTGA-GAAGACG -3') and reverse (5'- CGACGTAC-GTGCAGTCCA -3') and for the second amplification round, the primers were as forward (5'-ACCGGTGCTGAGAAGACG-3') and reverse

(5'-CGACGTACGTGCAGTCCA-3'). Primers synthesized by Bioneer Company (Korea).

PCR

A 700 bp sequence within the ITS1 fragment was amplified by PCR following the protocol previously described (14). Total volume of reaction was 15µl containing 1.5µl DNA template, 5µl distilled water, 10pmol of each primers (Forward and Reverse), and 7.5µl master mix (amplicon). The negative control was the mixture reaction without any DNA template. The PCR products were separated in 1.5% agarose gel using Simply Safe (Eurx, Cat. No. E4600-01) (Fig. 2).

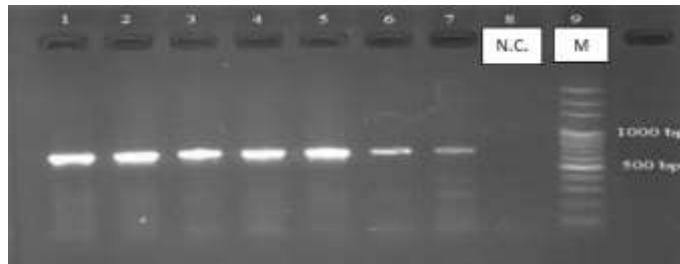


Fig. 2: Conventional PCR pattern of fascioliasis patients (positive for egg and antibody). Lane 1: Positive control, Lane 2, 3, 4, 5: Stool sample Lane 6, 7: Urine sample. Lane 8: Negative control, Lane 9: 100bp DNA ladder

Nested PCR

ITS1 fragment (about 430 bp) was amplified by nested PCR. Reactions were performed in the total volume of 15µl containing 1.5µL of 1/160 diluted first-round PCR product, 5µl distilled water, 10pmol of each primers (Forward and Reverse), and 7.5µl master mix (amplicon). The negative control was the mixture reaction without any DNA template. Three negative controls and one positive control (DNA extracted from *Fasciola* sp.) were included in each round.

The temperature profile for both rounds of amplification was as follows: an initiation 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec (denaturation), 60 °C for 30 sec (annealing), 72 °C for 30 sec (extension) and a final extension at 72 °C for 5 min followed by cooling at 4 °C. 5µL aliquot of nested PCR product was electrophoresed on 1.5% agarose gel and visualized using ultraviolet light after staining with Simply Safe (Eurx, Cat. No. E4600-01) (Fig. 3).



Fig. 3: Nested PCR pattern of fascioliasis patients (negative for egg, positive for antibody). Lane 1: Negative control, Lane 2,3,4: Stool sample, Lane 6,7,8: Urine sample, Lane 9: Positive control, Lane 10: 100bp DNA ladder

Sequencing

To confirm the results of nested PCR, 20 positive products including 5 parasitological positive stool, 10 parasitological negative stool and 5 urine positive samples were randomly selected and prepared for sequencing. These nested PCR products were purified and sequenced unidirectional using the forward primer used in the second-round PCR. Sequence results were aligned and edited by BioEdit version 7.2.5, and compared with GenBank reference sequences from the region, related to *Fasciola* spp. using the BLAST query facility.

Analytical sensitivity and specificity of nested PCR

In order to assess the specificity of the nested PCR, genomic DNA was extracted from positive stool and urine samples for strongyloidiasis, toxocariasis, trichostrongylosis, toxoplasmosis and hydatidosis using a DNA extraction kit from Bi-oneer according to the manufacturer's protocol. Nested PCR of these dilutions was carried out in several runs as mentioned above.

Furthermore, the sensitivity of the designed protocol was tested by using genomic DNA extracted from 25 negative stool and 10 negative urine samples (Table 1).

Table 1: Profile of *Fasciola* spp. sequenced in this study

Isolate	sample	Location	Serological method	Parasitological method	Conventional PCR	Nested PCR	Genus	Accession number
IR	Stool	Mianeh	+	+	+	+	<i>F. gigantica</i>	MF372919
IH 3	Stool	Mazandaran	+	+	+	+	<i>F. hepatica</i>	MF187486
IH 4	Urine	Tehran	+	-	-	+	<i>F. hepatica</i>	MF187487
IH 5	Stool	Behbahan	+	-	-	+	<i>F. hepatica</i>	MF187488
IH6	Stool	Tehran	+	-	-	+	<i>F. hepatica</i>	MF187489
IH7	Urine	Kermanshah	+	-	+	+	<i>F. hepatica</i>	MF187490
IH8	Stool	Tehran	+	-	-	+	<i>F. hepatica</i>	MF187491
IH 9	Stool	Mazandaran	+	-	-	+	<i>F. hepatica</i>	MF187492
IH 10	Urine	Khorasan	+	-	-	+	<i>F. hepatica</i>	MF187493
IH 11	Stool	Mazandaran	+	-	-	+	<i>F. hepatica</i>	MF187494
IH 12	Urine	Borujerd	+	-	+	+	<i>F. hepatica</i>	MF187495
IH 13	Stool	Kerman	+	+	+	+	<i>F. hepatica</i>	MF187496
IH 14	Stool	Mazandaran	+	+	+	+	<i>F. hepatica</i>	MF187494
IH 15	Stool	Khorasan	+	-	-	+	<i>F. hepatica</i>	MF187498
IH 16	Stool	Tehran	+	-	-	+	<i>F. hepatica</i>	MF187499
IH 17	Stool	Mazandaran	+	+	+	+	<i>F. hepatica</i>	MF187500
IH 19	Stool	Tehran	+	-	-	+	<i>F. hepatica</i>	MF187501
IH 20	Urine	Borujerd	+	-	-	+	<i>F. hepatica</i>	MF187502
IH 21	Stool	Ardabil	+	-	-	+	<i>F. hepatica</i>	MF187503
IH 22	Stool	Tehran	+	-	-	+	<i>F. hepatica</i>	MF187504

Statistical analysis

Statistical analysis was done using SPSS version 22 (Chicago, IL, USA). Chi-square test was used for data analyzing. Cut-off was calculated as Mean \pm 3SD.

Results

Out of 206 examined cases, 24.8% were seropositive for fascioliasis as previously reported (11). Of these patients, 25 seropositive cases were

randomly assigned to the study. Among them, *Fasciola* eggs were found in 5 (20%) of cases by parasitological methods (formalin ether concentration technique). One of these cases reported previously (14). Conventional PCR was positive in 5(20%) of stool and 3(30%) of urine in seropositive samples for fascioliasis. Also 24(96%) and 9(90%) of stool and urine samples were positive according to Nested PCR, respectively (Table 2).

All patients had a history of eating raw vegetables or drinking unsafe water. In these patients, two patients were asymptomatic, 68.6% of patients reported liver pain, and 23.5% of patients complained of cutaneous symptoms including itching and rash. Pulmonary symptoms were recorded in 25.5% of patients. All of them showed more or

less peripheral eosinophilia, ranging from 6% to 66 % (11).

Stool and urine samples of patients with strongyloidiasis, toxocariasis, trichostrongylosis, toxoplasmosis and hydatidosis were negative for nested-PCR. The nested-PCR assay could detect the target segmented DNA of *Fasciola* spp. in stool and urine of patients.

Table 2: Comparison of the results obtained by parasitological and molecular methods for detection of *Fasciola* spp. in serological positive patients (P=Positive, N=Negative)

	Seropositive group (n = 25)						Negative control group (n = 25)					
	Conventional PCR		Nested PCR		Parasitology		Conventional PCR		Nested PCR		Parasitology	
Stool	P	N	P	N	P	N	P	N	P	N	P	N
	5	20	24	1	5	20	0	25	0	25	0	25
	Seropositive group (n = 10)						Negative control group (n = 10)					
Urine	P	N	P	N	P	N	P	N	P	N	P	N
	3	7	9	1	-	-	0	10	0	10	-	-

Discussion

The human fascioliasis in Iran requires constant monitoring due to its significant characteristics and habitats. In 1989 and 1999, Iran experienced two major human fascioliasis outbreaks that affected more than 15,000 individuals (3). However, the recent study showed that the prevalence of fascioliasis as 0.4% using parasitological methods and 1.2% using serological methods indicating the region as a hypoendemic (15). WHO believes that Iran has serious problems with fascioliasis (16). Some new reports more precautions to monitor disease in different regions (11, 17, 18).

Fasciola hepatica has economic and public health implications, especially in subtropical regions and temperate climates. In the hepatic phase, young flukes first migrate to the liver parenchyma. After 6-7 weeks, they enter into the bile ducts and become sexually mature (19). In this study, 24.8% of patients were seropositive using serological test. Although golden standard is a fecal exam for diagnosis of fascioliasis, documented issues are less susceptible (8, 20), however, many studies have so far confirmed the outstanding role of serologic tests for detecting fascioliasis (7, 12, 21). Therefore, accurate diagnosis and treatment

of fascioliasis is necessary. Conventional parasitological methods are gold standard in diagnosis of fascioliasis but are not sufficiently sensitive to detect of fascioliasis, and repeated examinations of stools over many consecutive days are necessary. The serological method has been recognized as being more sensitive than parasitological methods in the diagnosis of fascioliasis. The sensitivity of serological method is higher than parasitological methods; nevertheless, its efficacy was not 100%, implying the need for new methods with higher sensitivity. Although parasitological methods are gold standard in diagnosis of fascioliasis, they are not sensitive enough to detect fascioliasis (22, 23). Detection of biomarkers in the blood circulation like other body fluids is now widely used in medical research (10). Of the various types of diagnostic tools currently available for detection of cfDNA in a range of clinical settings (24, 25). CfDNA contains DNA fragments of extracellular in the circulation which its appears in the blood cfDNA as well as in saliva (26), urine (27, 28), stool (29), sputum (30), cerebrospinal fluid (CSF) (31), peritoneal fluid (32), synovial fluid (33), and lymph fluid (34). Despite the various hypotheses offered, the exact origin of the cfDNA is unknown (24). About 20 mg/ml

cfDNA consisting of both mtDNA and nuclear DNA can be detected in the circulation of ordinary person (25). In circulation, cfDNA will be destroyed by nucleases (35). In primary experimental studies in animals, most of the products of cfDNA are cleared by liver and kidney (36). In addition, the parts of cfDNA available in the circulation, appear in the urine and detectable by methods of genetic analysis (28).

Therefore, it is possible to detect cfDNA components in circulation and urinary components. Nowadays, various methods for detecting host parasites are based on nucleic acid enhancement experiments (NAATs) to detect cfDNA from the parasite in host body fluids, especially acceptable clinical specimens such as urine and stool. Today, cfDNA is widely used to detect parasites such as *Leishmania*, *Plasmodium*, *Trypanosoma*, *Schistosoma* and *Wuchereria* spp. (37-40). These are indirect contact with the circulatory system, there is a possibility that the entire DNA of parasites in intact or in parts include cfDNA that constantly and fairly to the abundance in circulation can be poured from the blood and other body fluids extraction. The cfDNA parasite can enter the bloodstream and then distribute it in other body fluids. It can also be released directly in each body fluid depending on the location of the parasite in the host. The exact source and distribution of cfDNA in the host is still not fully understood, but can be directly active from the parasite or its products, through passive diffusion and secretion (37).

The cfDNA can detect a severe infection or an infected host in the absence of unknown parasites, which may otherwise be undetectable. Early detection of infection by using cfDNA can lead to timely treatment. This indicates an additional source of DNA in the feces and urine detectable by PCR, which could be cfDNA or parasitic fragments not detected by a microscope (40). PCR-based methods have shown variable sensitivity for detection of *Fasciola* in fecal samples. For detection DNA of worms in stool samples by PCR-based techniques, optimization of DNA isolation method is essential, because of existence of large amounts of PCR-inhibitory substances in

fecal samples such as bacterial proteases, nucleases, cell debris, bile acids, and so for, and also the presence of a thick and complex cuticle that covers the worms (41, 42). In this method, routine commercial DNA extraction kits from stool (Qia gen) were used. The DNA extraction process is a critical step affecting the efficacy of molecular methods. The ITS1 gene was used as a conserved target for the *Fasciola* spp. in specific nested PCR. Nested PCR not only identified all parasitological positive samples, but also additional samples not detected by parasitological methods. Considering parasitological methods as the gold standard, diagnostic sensitivity and specificity of nested PCR was as high as parasitological methods. Nested PCR was the only method that did not miss any seropositive cases compared with other methods except one specimen.

This assay detected DNA released from *Fasciola* sp. in 24 (96%) stool samples and 9 (90%) urine samples with a specificity of 100%. Different sensitivity and specificity have been reported in other studies according to sample size, DNA extraction methods, and parasitic load of stool samples. Studies on other microorganisms including *Toxoplasma gondii* and *Histomonas meleagridis* have also reported higher sensitivity of nested PCR compared with real-time PCR (43, 44).

Conclusion

Detection of *Fasciola* cfDNA in stool and urine samples has high accuracy, so might be used as for the diagnosis of human Fascioliasis infection.

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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