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Prevalence of bovine fascioliasis in a new-emerging focus of human fascioliasis in BoyerAhmad district, southwest of Iran

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ABSTRACT

Background: The prevalence of fascioliasis in a population of cattle in a new-emerging focus of human fascioliasis in Boyer-Ahmad District, in the southwest of Iran, was investigated, using an ELISA system and coprological methods.
Methods: Blood, as well as stool samples, were collected from 150 dairy cattle consisted of 82 (54.7%) males and 68 (45.3%) females, aged from 1 to 8 years, selected from different pastures. The stool samples were evaluated by direct wet mount microscopic examination as well as sedimentation methods. Moreover, modified Telemann was used for detecting of *Fasciola* eggs. Anti-*Fasciola* antibodies in the cattle sera samples were determined, using a *Fasciola* excretory-secretory antigen-specific ELISA.
Results: Anti-*Fasciola* antibodies were detected in 64 (42.4%) of the subjects by ELISA. Out of 64 seropositive cases, 29 (45.3%) were female and 35 (54.6%) were male. No significant association was found between sex and seropositivity to fascioliasis. Considering the age of the cattle, the highest prevalence of fascioliasis (23%) was found in the 5-year old age group. However, the difference between the age of the cattle and seropositivity to fascioliasis was not statistically significant ($P > 0.05$). *Fasciola* eggs were not detected in fecal samples of any of the studied cattle, while Paramphistomide, as well as *Dicrocoelium dendriticum* eggs, were detected in 6 (4%) and 17 (11.3%) of the subjects, respectively.
Conclusion: Findings of the current study provide basic information about bovine fascioliasis in a new focus of human fascioliasis in Iran, which is needed for effective control of this parasitic infection in such areas.

1. Introduction

Fascioliasis is an important helminthic zoonotic disease caused by liver flukes of the genus *Fasciola hepatica* and *Fasciola gigantica*. The disease occurs in some of the mammalian animal hosts and humans worldwide. Fascioliasis is considered as an emerging/reemerging and also a neglected tropical and foodborne disease, with the widest known distribution that has partly been related to climate changes [1]. Fascioliasis is common in livestock including sheep, goat, and cattle and accounted for a major economic impact on the global livestock industry as a result of liver condemnations lost production as well as mortality. Decreased milk yield, diarrhea, weight loss, infertility, and occasional death are all the effects of *Fasciola* infection in livestock [2]. Moreover, fascioliasis is an important human food-borne disease and harmful to public health. Human fascioliasis is endemic in more than 60 countries in the world, where more than 17 million individuals are infected and about 180 million individuals at risk of this infection [3].

Human become infected through drinking contaminated water with metacercaria or eating contaminated aquatic plants. Infected livestock can be a source of human infection in endemic areas. Both animal and human fascioliasis, caused by both species of the fluke, *F. hepatica*, and *F. gigantica*, are major economic and health problem in Iran [4,5]. In a recent review of animal fascioliasis in Iran, the prevalence of animal infections was reported to be as 6.2%, including 4.2% in sheep, 9% in cattle and 3.1% in goat. While the lowest prevalence (1.8%) was reported in central Iran, the highest prevalence (11.8%) was reported in the north of the country, where the rate of human fascioliasis is also high [4]. In Guilan province, in the north of Iran, two major outbreaks of human fascioliasis in 1987 and 1997 affected more than 15,000 people. Since then, cases of human fascioliasis are continuously being

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reported from this area [5].

Recently, human fascioliasis has emerged in other areas of Iran, including in Kohgiluyeh and Boyer-Ahmad province southwest of the country [6–8]. A seroepidemiological study showed that 1.86% of the populations are seropositive for fascioliasis in this area [7]. Likewise, a recent study demonstrated anti-*Fasciola* antibodies in sera of 2.4% of nomads living in the same area [9]. Resident of this area used to consume aquatic plants including *Nasturtium microphyllum* (local name Bakaloo), *Mentha logifolia* (local name, Pooneh) and spearmint and these plants are considered as the main source of infection in this area [6,7]. Infected sheep and cattle are responsible of maintaining the *Fasciola* life cycle in endemic area, and this may pose a permanent risk for humans' infection.

The recent studies regarding the prevalence of human fascioliasis in a new focus of fascioliasis in Kohgiluyeh and Boyer-Ahmad Province in the southwest of Iran guided us to determine the prevalence features of this disease in cattle as one of the main animal hosts of fascioliasis in this area.

2. Materials and methods

2.1. Ethical statement

The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (SUMS), (Reference No. IR.SUMS.REC.1398.052).

2.2. Study area

The study was conducted in Boyerahmad district, in Kohgiluyeh and Boyer-Ahmad province which is located in the southwest of Iran with geographical coordinates between latitudes: 30.67 N and longitudes: 51.60 E (Fig. 1). The annual average temperature and rainfall of the area are 15.2 °C and 864.9 mm. Located in the Zagros Mountains range, Boyer-Ahmad district has a cold winter and moderate summer and the mountain is covered with wild pistachio and oak forests. Its geographical condition and climate are suitable for agriculture and animal husbandry. The moderate climate and large pastures for ruminants provide an appropriate condition for transmission of parasitic infection, including fascioliasis [6–11]. Cows are always housed in the region and fed on grass pasture and provide manure for soil fertilization. More than half of the farms within the area of the study had less than four animals.

2.3. Sample collection

Blood, as well as stool samples, were collected, during November 2018 to February 2019, from 150 dairy cattle selected from different pastures, using convenient randomized sampling method. Blood samples were centrifuged and sera were stored at −20 °C until tested. Fecal samples were collected from the cows' rectum, using appropriate disposable gloves. During sampling, a data collection sheet was used to record information such as age, sex, pregnancy, type of nutrition, disease, and place of sampling for each cow.

2.4. Parasitological evaluation of fecal samples

The stool samples were evaluated by direct wet mount microscopic examination as well as sedimentation methods. To prepare a direct wet mount, 1 g of the specimen was dissolved in one drop of 0.9% saline and was examined for parasites ova by light microscope, using 10X and 40X magnifications. Moreover, modified Telemann's method was used for detecting of *Fasciola* eggs in the stool samples, as previously described [6]. Briefly, three grams of stool sample was dissolved in 10 mL of 0.9% saline in a 15 mL tube and the solution was passed through four layers and then two layers of gauze. Samples were centrifuged (800 *g* for one minute), and the supernatant was discarded. The sediment was mixed with 5 mL of 15% hydrochloric acid. Then, 5 mL of ethyl acetate was added and the tube was gently shaken and centrifuged as before. The supernatant was discarded and the sediment was used to prepare a direct smear slide. Slides were examined under the light microscope, using 10X and 40X magnifications to detect *Fasciola* eggs. Moreover, a staining method, using methylene blue, was applied by adding one drop of methylene blue solution to the sediment to differentiate *Fasciola* from *Paramphistoma* eggs. Using this contrast stain, eggs of *Fasciola* showing yellowish color while eggs of paramphistomids stain. [12]

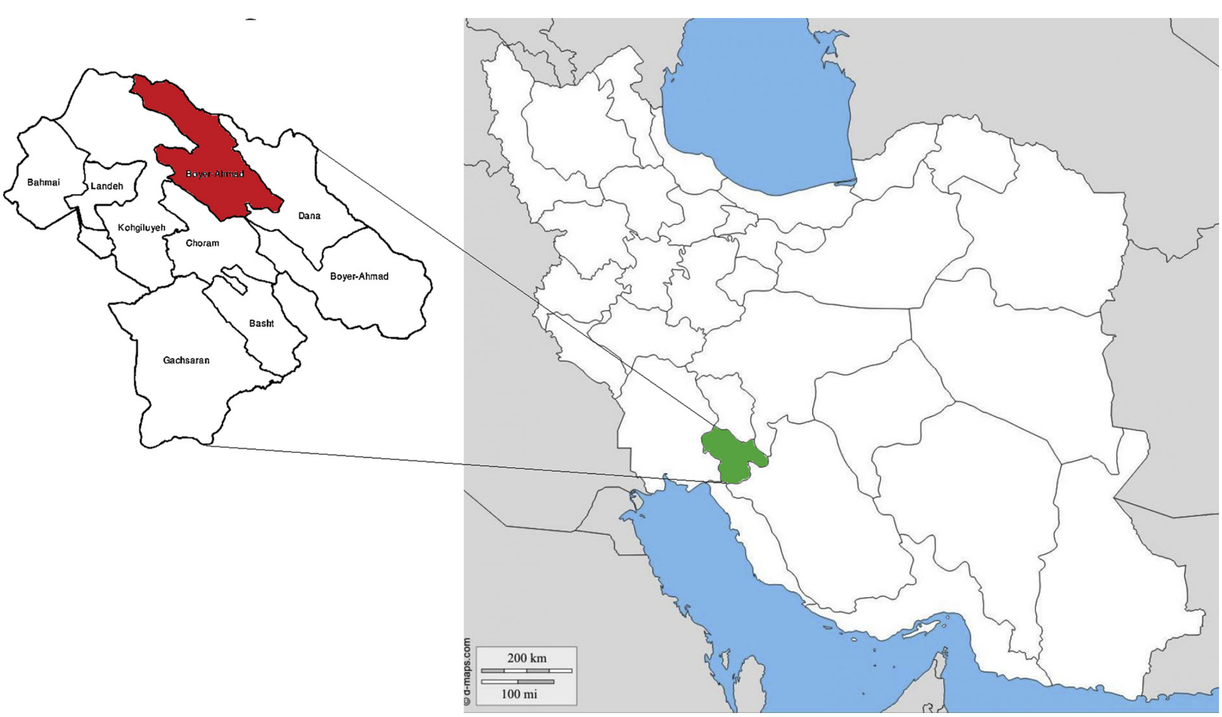

Fig. 1. Map of Iran showing the study area.

Table 1
Characteristics and relative seropositivity to *Fasciola* in cattle in Kohgiluyeh and Boyer-Ahmad Province in the southwest of Ira.

Characteristics	Frequency (No.)	Percent (%)	Positive for anti- *Fasciola* antibodies		
			Frequency (No.)	Percent (%)	
Gender					$P > 0.05$
Male	82	54.7	35	42.7%	
Female	68	45.3	29	42.6%	
Age					$P > 0.05$
1	32	48	13	40.6	
2	10	6.67	2	20	
3	13	8.67	5	38	
4	14	9.3	6	42.8	
5	35	23.3	16	45	
6	30	20	14	46	
7	15	10	7	46.6	
8	1	0.67	1	100	
***Dicrocoelium* eggs (in stool)**					$P > 0.05$
Positive	17	11.3	6	35.2	
Negative	133	88.67	11	8.27	
Paramphistomidae eggs (in stool)					
Positive	6	4	2	33.3	
Negative	144	96	4	2.77	

2.5. Preparation of Fasciola excretory-secretory (ES) antigens

Intact living adult *Fasciola* worms were obtained from the bile ducts of infected sheep from the slaughterhouse. The worms were washed five times with PBS and transferred into culture flasks, containing RPMI 1640 medium and incubated at 37 °C for 15 h. Then, the flukes were removed and the remaining media containing the ES antigens was centrifuged (13,000 *g* for 30 min). The supernatant was collected and dialyzed against PBS. The protein content of the sample was estimated by the Bradford method and the antigen was kept at −20 °C until use [13].

2.6. Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-Fasciola antibodies

Anti-*Fasciola* antibodies in the cattle sera samples were determined, using a *Fasciola* excretory-secretory antigen-specific ELISA, as previously described with minor modifications. ELISA was carried out in flat bottom 96 well microplates (Nunc, Nagle, Denmark). The plates were coated with 5 μg/mL of ES antigen (100 μg/well) in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated at 4 °C overnight. Plates were washed 5 times in phosphate-buffered saline-Tween 20 (PBST, pH 7.4 containing 0.05% Tween 20). Blocking was performed with 3% skimmed milk in PBST for 1 h. Plates were washed as before and 100 μL of serum sample (1/100 dilution in PBST) was added to each well and incubated for 2 h at room temperature. The plates were washed as before and 100 μL aliquots of horseradish peroxidase-conjugated anti-cow antibody (at a 1/1000 dilution in PBST) was added to the plates and incubated in room temperature for 1 h. After washing as before, the plates were incubated with chromogen/substrate (100 μL/well of OPD, 0.025% H_2O_2 in 0.1 M citrate buffer, pH 5) and, after 30 min, the absorbance at 490 nm was measured, using a microplate reader. Negative control (obtained from healthy cattle in Shiraz Faculty of Veterinary Medicine, with no history or sign of fascioliasis post-slaughter examinations) and positive control sera (8 positive bovine sera from confirmed fascioliasis cases) were included in each run of ELISA. The cut point was set at 2 SD above the mean of negative controls.

2.7. Statistical analysis

SPSS (version 18) was used for the analysis of the data. Descriptive statistics were performed to indicate the frequencies of the interested variables. The association between prevalence of fascioliasis and qualitative variables were assessed by Chi-square test. The significance level was defined as 0.05.

3. Results

Subjects of the study were 150 dairy cattle consisted of 82 (54.7%) males and 68 (45.3%) females. Their ages ranged from 1 to 6 years. The most frequent age (23.3%) was 5 years old.

Anti-*Fasciola* antibodies were detected in 64 (42.4%) of the subjects by ELISA. Out of 64 seropositive cases, 29 (45.3%) were female and 35 (54.6%) were male. No significant association was found between sex and seropositivity to fascioliasis. Considering the age of the subjects, the highest prevalence of fascioliasis (46.6%) was found in the 7-year old age group, although, in the eight-year-old group, only one sample was taken which was positive. The difference between the age of the cattle and seropositivity to fascioliasis was not statistically significant ($P > 0.05$). As there were no substantial differences in feeding types of cattle, no significant differences were found between seropositivity to fascioliasis and feeding type.

Fasciola eggs were not detected in any of fecal samples examined by either sedimentation or modified Telemann methods, whereas Paramphistomidae, as well as *Dicrocoelium dendriticum* eggs were detected in 6 (4%) and 17 (11.3%) of the subjects, respectively. Table 1 shows the characteristics of cattle and related infection in this study.

4. Discussion

Given the scarcity of currently available data about the prevalence of fascioliasis in livestock in Iran, the current study uses ELISA as well as coprological methods to define the prevalence of fascioliasis in cattle in Kohgiluyeh and Boyer-Ahmad Province in southwestern Iran. Findings of the study revealed a seroprevalence rate of 42.4% for fascioliasis in the studied cattle in the area. In a study conducted by Moshfe and others about fascioliasis in slaughtered animals in the same area, 12.5% of cattle were reported to be infected by *Fasciola* spp. [14]. In the same region, human fascioliasis has been investigated in our two previous studies, conducted in 2011 and 2018respectively, where the seroprevalence of fascioliasis in human was estimated at 1.86% in people living in the areas and 2.6% in nomad population [7,9]. In a recent population-based study in the same area, from 1050 inhabitants of 50 randomly selected villages, *Fasciola* eggs were detected in the stool of two (0.19%) individuals [6].

Moderate temperatures, about 800 mm rainfall during the year, and large pasture for ruminants provided ideal climatic conditions for transmission and establishment of fascioliasis in this area [15]. Molecular studies demonstrated that both *F. hepatica* and *F. gigantica* are infecting cattle, goats, and sheep in this area. High prevalence of fascioliasis in animals in this region favor the distribution of the infection, and food habitat of people for eating the uncooked aquatic plants ensures the endemicity of human fascioliasis in this area.

Cattle are one of the main important animal hosts of fascioliasis. The rate of fascioliasis in cattle has been reported to be 30–80% in developing countries [16]. The seroprevalence rate of fascioliasis in cattle varies in different areas of the world including 3.40%, in Switzerland, 37% in India, 8.9–25%, in Sweden, 6.2–52% in Germany [2,17–21]. In a study in Pakistan, using ELISA with *Fasciola* ES antigens, anti-*Fasciola* antibodies were detected in 38% of cattle [22]. The seroprevalence of *F. hepatica* infection in a population of commercial dairy herds in England and Wales was estimated at 72% and 84% respectively [23]. An epidemiological study of *F. hepatica* in cattle was implemented in the north-central region of Portugal where anti-*Fasciola* antibodies were detected in 11–48% of cattle [24]. The seroprevalence of fascioliasis in sheep and cattle from the Altiplano of Bolivia, which is considered as the main focus of human fascioliasis in the world, was reported to be 89% in sheep and 58% in cattle [25]. In a study by Kuerpick and others, a total of 20,749 bulk tank milk samples from all over Germany, corresponding to 20.9% of all German dairy herds, were tested by ELISA to detect anti-*Fasciola* antibodies. The mean seroprevalence was reported to be 23.6%, varied from 2.6% to 38.4% in different German federal states [18]. The herd-level seroprevalence of *Fasciola* infection in dairy Polish and Swedish dairy cattle was estimated at 79.6% and 25%, respectively [21,26].

Findings of the current study revealed that 42.4% of the cattle are seropositive for fascioliasis in a previously recognized focus of human fascioliasis in the southwest of Iran. This indicates that fascioliasis is causing significant financial losses due to a decrease in dairy productions. Furthermore, a severe infestation might directly or indirectly lead to mortality through triggering or intensifying disease.

It is difficult to compare the prevalence of bovine fascioliasis in our study with those studies from other countries as different methods such as coprological antigen detection, antigen-based serum ELISA, bulk milk tank ELISA, post-slaughter inspections might be used in different studies conducted in different areas of the world. Nevertheless, one common aspect in all of these studies is that they generally show the relative incidence of *Fasciola* infection in a given region.

The samples tested in the current study were convenience samples, which were selected randomly from different dairy herds and the results can be considered a good estimate of seroprevalence levels in the regional dairy herd.

The presence of anti-*Fasciola* antibodies reflects previous exposure to the flukes. Therefore, the cattle seropositivity does not necessarily indicate active fascioliasis, but it does point out that animals have been exposed to the parasite. Moreover, the rate of seropositivity to *Fasciola* is usually higher than the rate obtained from slaughterhouse inspection, or fecal sample examination, as these show the active fascioliasis. This is the main reason that the prevalence of fascioliasis in our study is higher than those studies which are based on slaughterhouse examination of slaughtered cattle. As a few examples, in a study on bovine fascioliasis in Rudsar, North of Iran, a total of 680 slaughtered cattle were examined for the presence of *F. hepatica* and 137 cattle (20.14%) were found to be positive [27]. Analysis of fascioliasis in slaughtered cattle in Tehran, capital of Iran, during 2015–2018 revealed that 0.25% of cattle were infected by *Fasciola* spp. [28] An abattoir survey of fascioliasis in Alborz Province, southern Iran, revealed an infection rate of 2.09% in slaughtered cattle [29].

In the present study, ELISA based on ES antigens of *Fasciola* has been used to detect anti-*Fasciola* antibodies in cattle serum. The sensitivity and specificity of ES antigen for detection of *Fasciola* antibodies in bovine sera have been reported to be 85–95% and 90–95%, respectively [30–32].

Fasciola eggs were not detected in the cattle stool samples in our study. This is somewhat unexpected. The lack of eggs in the stool samples of positive cases may be due to the incapability of *Fasciola* to produce eggs, lack of adaptation of the fluke to the host, or encapsulation of eggs in the liver granuloma, and low egg releasing because of low infection burden or old infection. Furthermore, intermittent egg production and cessation of egg shedding in the advanced chronic phase of fascioliasis is not uncommon.

5. Conclusion

Fascioliasis is a common parasite in cattle in the southwest of Iran. Findings of the current study provide basic information about bovine fascioliasis in a new focus of human fascioliasis in Iran, which is needed for effective control of this parasitic infection in such areas.

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Declaration of Competing Interest

None to declare.

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