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Molecular Identification and Prevalence of *Fasciola gigantica* in Cattle and Buffaloes of Punjab, Pakistan

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KA presented the concept. MK, HSZH and SF curated data. GN and KA performed formal analysis and software. MK, KA and SF planned methodology. KA, MK, SF and GN validated the study. KA, MK, HSZH and SF wrote the manuscript.

Key words
Molecular characterization, Morphology, ITS-I, Copro-ELISA, Fasciolosis, Pakistan

ABSTRACT

Fasciolosis is a food- and water-borne trematodiosis caused by *Fasciola hepatica* and *F. gigantica*. Hybridization between the two species has been reported in Punjab. Therefore, this study aimed at morphological and molecular characterization of *Fasciola gigantica* and its prevalence in cattle and buffaloes of Punjab province, Pakistan. A total of 100 fluke specimens were collected from cattle and buffaloes slaughtered at abattoirs and were classified as *Fasciola spp*. using morphological characters. Of these species, 62 flukes of 31 populations were identified with molecular analysis using the ITS-I marker. Copro-ELISA assay was performed to find the prevalence of fasciolosis. Morphological and molecular analysis showed that *Fasciola* spp. species formed a moderately supported monophyletic clade with *F. gigantica*. The phylogenetic analysis showed conclusive evidence for the clade containing Indian and Chinese *F. gigantica*. The copro-ELISA result showed that the diagnostic accuracy of the test was 89.22% with 100% sensitivity and 82% specificity. The overall prevalence of fasciolosis was 42.7%, and infection was significantly ($p<0.001$) higher in cattle 25.3%, as compared to 17.4% in buffaloes. The fasciolosis was significantly ($p=0.02$) higher in females (24.6%) compared to males (18.0%), and animals belonging to >3-6 years of age group showed the highest prevalence of 30.3% than other age groups. In conclusion, the use of morphological techniques, complemented by molecular techniques is recommended, in endemic areas where the two species are co-endemic. Furthermore, the immunodetection assays are more sensitive to find the epidemiological status of disease as compared to conventional microscopic fecal examination.

INTRODUCTION

Fasciolosis is a parasitic zoonosis resulting from exposure to *Fasciola hepatica* or *F. gigantica* liver flukes. Fasciolosis is endemic throughout the world infecting 600 million domestic ruminants, causing major economic losses estimated to be US$3 billion per annum; some 17 million people are also infected in 61 countries with 180 million at risk of infection and the burden of disease due to fasciolosis is estimated at 90,000 disability-adjusted life years (Mas-Coma *et al*., 2019). Fasciolosis has also been reported in all part of Pakistan, with an overlap of the two species *F. gigantica* and *F. hepatica* (Afshan *et al*., 2014a). The climate change and anthropogenic environment modifications are influencing the fascioliasis risk by anthropogenic activities and climate change (Afshan *et al*., 2014b, 2022). The prevalence of helminthes in different species of animals has been reported ranging from 21.41 to 92% in Pakistan. The problem of fascioliasis has been diagnosed in all areas of the Punjab but is a main problem in swampy areas enriched with the intermediate hosts, like snails and is one of the main constraints in development of a profitable livestock industry (Farooq *et al*., 2015; McManus, 2020).

Fasciola species tend to be sympatric in several sub-tropical and hot temperate climes, particularly in Asia and Africa (Kalu, 2015). This overlapping prevalence has resulted in disagreements over the taxonomic differentiation of *Fasciola* species in Far Eastern countries, where the exitance of intermediate form of *Fasciola* has

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been documented (Seid and Melese, 2018), and frequent in Asiatic countries (Yakhchali *et al*., 2015). Morphological criteria such as body size and shape are among the traditional and important methods to distinguish between the 2 species (Rouhani *et al*., 2017). However, relying just on morphometry is insufficient because the morphological characteristics of adult worms and eggs are impacted by a variety of parameters, including the host type, parasite age, sample fixation, and infection severity.

However, because of considerable variation and overlap in measurements between *Fasciola* species such phenotypic criteria are unreliable for specific identification and differentiation between *Fasciola* species (Ahmad *et al*., 2021). Both species were shown to be spermic diploid and capable of meiosis. Flukes which have intermediate morphological properties of both *F. hepatica and F. gigantica* have created uncertainty, provoking a rise in the cumulative usage of molecular (Villa-Mancera *et al*., 2021) and morphometric methods to differentiate the species. Because morphological methods have limits, numerous molecular approaches based on different molecular targets have been developed to differentiate *Fasciola* species. Because of the repeated sequence and the existence of variable sections flanked by more conserved region, nuclear rDNA is particularly relevant for molecular investigations. The nuclear rDNA sequences of ITS-I and II, which are found between the 18S, 5.8S, and 28S coding areas, have been employed for species-level molecular identification (Amer *et al*., 2016).

Epidemiological studies on fasciolosis mainly relay on fecal examination or on inspecting animal directly at slaughterhouses, which is time consuming and laborious process and is insensitive in case of low parasite burden. Recent approaches are based on immunological techniques that tend to improve the accuracy and sensitivity of fluke detection in feces of livestock (Martinez-Sernandez *et al*., 2016). The potential usage of a *Fasciola* coproantigen test has been described by its virtue to detect very low number flukes burden, for example, by one fluke, or five metacercarial cysts. Moreover, copro-ELISA is more sensitive in recognizing active fasciolosis than of methods used for detecting excretory/secretory antigens in serum. The experimental infection has investigated the high sensitivity and specificity of coproantigen-based ELISA (Cwiklinski *et al*., 2019).

However, a very few researchers tend to use immune-serological techniques (Afshan *et al*., 2014b, 2017, 2022). Lack of widely accepted and sensitive diagnostic tool is reason behind the underestimation of these trematodiases incidence. There is a scarcity of useful data on the phenotyping and the molecular characterization of *Fasciola* spp. in Pakistan's large ruminants. Therefore, the present study aimed to identify accurate fasciolids species based on morphological and molecular markers and to determine the distribution of fasciolosis by copro-ELISA in Punjab, Province Pakistan.

MATERIALS AND METHODS

Study area

The Punjab province of Pakistan was the subject of research and accounts for 25.8% of Pakistan's total land area. Adult and mature liver flukes were obtained from the bile duct of buffaloes and cattle of either sex that were slaughtered at the Rawalpindi, Gujrat, Khushab and Kharian slaughterhouses between June 2019 to April 2020. Bovines are brought in from across the Punjab province to these slaughterhouses. This research included Sahiwal cattle, Nili Ravi, and Azi-kheli buffaloes of various ages and genders, mostly those kept on natural grazing and other seasonal fodders by using a random sampling procedure.

Collection of adult flukes

This study included 170 adult liver flukes from 130 infected cattle and buffaloes. The livers, gall bladders, and bile ducts of the slaughtered animals were inspected. The bile ducts were incised longitudinally through the gall bladder into the liver, and the parasites were extracted using fine forceps, taking all essential measures to prevent parasite damage. Only adult flukes from the collection were selected for further analysis, and they were determined as gravid due to the presence of many eggs in the uterus. To remove debris, each worm was rinsed twice in a 0.85% saline solution. *Fasciola* samples were transported to the laboratory and stored in 70% ethyl alcohol at room temperature for further analysis.

Morphometric analysis

A total 100 adult flukes from cattle (n=50) and buffaloes (n=50) were stained. Briefly, worms were washed with tap water for the removal of debris and fixed in Bouin's solution between two slides and stained with Grenacher's borax carmine and subsequently differentiated, dehydrated, and mounted in Canada balsam. Standard morphometric measurements were taken under the microscope as described by Periago *et al*. (2006).

Molecular analysis

The DNA was extracted from 31 populations (n=62) with the phenol chloroform technique (Sambrook *et al*., 1989). The ITS-I region of rDNA was amplified with a set of primers ITS-I Forward 5' GCGACCTGAAAATCTACTCTTACACAAGCG 3' and ITS-I-Reverse 5'

GACGTACGTATGGTCAAAGACCAGGTT 3'. The 25 µl PCR reaction mixtures consisted of 2 µl of PCR buffer (1×), 2 µl MgCl2 (25 mM), 2 µl of 2.5 mM dNTPs, 0.7 µl of primer mix (10 pmol/µl final concentration of each primer), 2 µl of gDNA, and 0.3 µl of Taq DNA polymerase (5 U/µl) and 16 µl ddH20. The thermo cycling conditions were 96 °C for 8 min followed by 39 cycles of 96 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 8 min. Aliquots (5 µl) of individual amplicons were examined on 1.5% (w/v) agarose gels and photographed using a GelDoc system.

PCR products were purified with WizPrepTM Gel/PCR Purification Mini kit (South Korea) and sequenced from DNA sequencing facility eurofins genomics (USA). Sequencer 5.4.6, Bio Edit software's were used to edit and align the ITS-I region of the *Fasciola* flukes. The unique sequences were submitted to GenBank, accession numbers were obtained and aligned with previously published NCBI GenBank rDNA reference sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. This analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 409 positions in the final dataset. The evolutionary distances were computed with Maximum Composite Likelihood method by using MEGA X (Kumar *et al*., 2018).

Copro-ELISA detection of fasciolosis

The fecal samples form from naturally infected cattle and buffaloes (n=499) grazing in the fields were collected. The liver and bile ducts of slaughtered animals were examined for *Fasciola* (gold standard of infection), and feces were collected from rectum. The positive (n=85) and negative (n=119) control fecal samples were collected. All fecal samples were mixed in distilled water at 1:1 ratio (3 g + 3mL) and subjected to centrifuge at 1,000g for 15 min. The supernatants were collected and analyzed for the presence of *Fasciola* coproantigens by ELISA.

ELISA was performed according to method described by Ahmad and Nizami (Ahmad and Nizami, 1998) with slight modifications. Fecal supernatant (50 µl/well) were coated in microtiter plates in a coating buffer, washed with PBS containing 0.1% Tween. Blocked with 150 µl of bovine serum albumin for 1hr and washed. Serially diluted 100 µl of *Fasciola* somatic antigen polyclonal antibodies (Afshan *et al*., 2021) were incubated for 2 h and washed. The alkaline phosphatase conjugated anti-rabbit IgG is diluted at 1:5000 dilution (Invitrogen Corporation, California, USA) and 100 µl of it was added to each well and incubated at room temperature for 2 h. The plates were washed, and reaction was developed by adding 100 µl of the substrate, para-nitrophenyle phosphate (PNPP) (Thermo Fisher Scientific Inc. Rockford, lL, USA). After 15-20 min the 50 µl of 3N NaOH was added to stop the reaction and OD was recorded at 405nm.

Statistical analysis

The diagnostic sensitivity, specificity, accuracy, and predictive values were calculated by using the online statistical software MedCalc (https://www.medcalc.org/calc/diagnostic_test.php). To compare prevalence among breed, age and sex categories Chi-square test were performed by using SPSS 22.0 statistical software. Significance was defined as $P < 0.05$. ROC curve values were computed by using GraphPad Prism (version 9).

Table I. Morphological measurements with descriptive statistical analysis of the *F. gigantica* collected from cattle (n=50) and buffaloes (n=50) of Punjab, Pakistan. The data shows range, mean and standard deviation values. All measurements are taken in millimeters (mm).

Parameters (mm)	*F. gigantica* buffaloes	*F. gigantica* cattle
Body length	34.46±0.51	20.1-41.3
Body width	5.84±0.09	6.01±0.17
Maximum diameter of oral sucker (OS max)	0.69 ± 0.14	0.84±0.02
Minimum diameter of oral sucker (OS min)	0.58±0.00	0.69±0.0
Maximum diameter of ventral sucker (VS max)	1.53±0.02	1.62±0.02
Minimum diameter of ventral sucker (VS min)	1.45±00	1.52±0.03
Distance between anterior end of body & VS(A-VS)	2.35±0.11	2.42±0.18
Distance between VS and posterior end of body (VS-P)	30.71±0.48	30.37±0.75
Body area (BA)	204.87±5.29	0.52±0.02
Oral sucker area (OSA)	0.49±0.01	207.29±9.0
Ventral sucker (VSA)	1.76±0.06	2.51±0.10
BL/BW ratio (BL/BW)	6.01±0.09	5.78±0.13

RESULTS

Morphometric analysis of F. gigantica

The morphological measurements of liver flukes from cattle and buffaloes slaughtered at different abattoirs of Punjab are grouped into *F. gigantica* like (Table I). The body length to width was 20.1 x 6.01 mm in cattle, and 34.46 x 5.84 mm in buffaloes. While the ratio of body length to width was 5.78±0.13 and 6.01±0.09 for cattle

Table II. Nucleotide variations in *F. gigantica* isolate A and B at fifteen different base positions collected from cattle and buffaloes of Punjab, Pakistan.

***Fasciola gigantica* Pakistan**	**Variable positions**														
	154	164	168	172	173	180	183	184	185	190	193	195	196	364	394
Fasciola gigantica isolate A (OM212803)	C	A	G	T	C	G	A	G	A	C	C	C	G	G	T
Fasciola gigantica isolate B (OM212804)	G	C	T	A	G	C	G	A	T	A	G	A	A	T	A

and buffaloes, respectively. The size of the fasciolids in buffaloes was found higher than cattle in most of the morphological measurements, however, the difference was not significant (P>0.05).

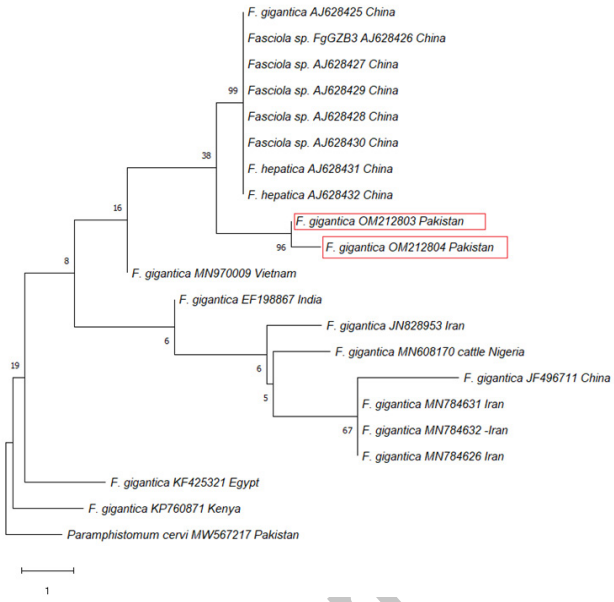

Fig. 1. Phylogenetic relationships of partial sequences of the first internal transcribed spacer of the nuclear ribosomal DNA of *Fasciola gigantica.* The tree with the highest log likelihood (-6673.04) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion option).

Molecular identification of F. gigantica

Adult fasciolids from 31 populations were sequenced and a 445-448 bp sequence of ITS-1 region of *Fasciola* species was obtained aligned for intraspecific variation. The intraspecific comparison of *Fasciola* sequences confirmed the existence of two genotypes i.e., which differed from each other at 15 base positions in the ITS-1 region (Table II). A summary of interspecific variations with nine reference sequences acquired from GenBank is given in Supplementary Table I.

The phylogenetic tree showed *Fasciola* haplotypes from geographically linked areas are close to each other (Fig. 1). *F. gigantica* isolate A (OM212803) and *F. gigantica* isolate B (OM212804) under the current study, fells in a clade with *Fasciola* species from China and Vietnam. The next clade belongs to *F. gigantica* from India and Iran. while Egypt and Kenya fluke groups are most distant. *Paramphistomum cervi* (MW567217) was selected as an outgroup, and the estimates of evolutionary divergence between sequences were 1.37-5.61 (Supplementary Table II).

Table III shows summary of indirect ELISA performed on samples using coproantigens. The copro-ELISA was performed on 85 true positive and 97 true negative coprological samples, and 22 coprological samples were detected false positive (79.44% positive predictive value). The area under curve (AUC) values was 0.991 (95% CI: 0.98-0.99; P<0.0001). A direct relation was observed between false positive (1-specificity) and true positive (sensitivity), further emphasizing the inverse relationship between sensitivity and specificity (Fig. 2A, B). The results showed a diagnostic accuracy of test 89.22% with 100% sensitivity and 82% specificity. The absorbance values denoted the specificity and sensitivity in the form of frequency and a large spread of positive controls was observed when negative and positive controls were plotted on a histogram (Fig. 2C, D).

Prevalence of fasciolosis

The overall prevalence of fasciolosis was 42.7%. The fasciolosis was found significantly (x^2 = 12.18, p<0.001) higher in cattle 25.3%, as compared to 17.4% in buffaloes. Similarly, the results showed fasciolosis was significantly (x^2 = 5.36, p=0.02) higher in female animals 24.6% compared to males 18.0%. Age-wis results showed the highest infection in >3-6 years of age group was 30.3%, while the 12.4% was found in 1–3 years age

group, respectively. However, the association was not statistically significant (x^2 =1.49; p>0.05). The scatter graph of individual OD values and cross-reactivity of the assay with other trematode parasites is plotted (Fig. 3A, B) and the association of disease risk factors is given in Supplementary Table III.

Table III. Diagnostic test performance by ROC curve and sensitivity and specificity values.

Parameters	Values	95% CI	P value
Copro-ELISA test true positive	85		
Copro-ELISA test true negative	97		
Copro-ELISA test false positive	22		
Copro-ELISA test false negative	0		
Area under the ROC curve			
Area under curve (AUC)	0.991	0.98-0.99	<0.0001
Std. Error	0.0044		
Diagnostic test evaluation			
Sensitivity	100%	95.68-100	<0.0001
Specificity	82%	73.59-87.46	
Positive predictive value	79.44%	70.83-86.01	
Negative predictive value	100%	96.19-100	
Positive likelihood ratio	5.41	3.71-7.89	
Accuracy of diagnostic test	89.22%	84.13-93.12	

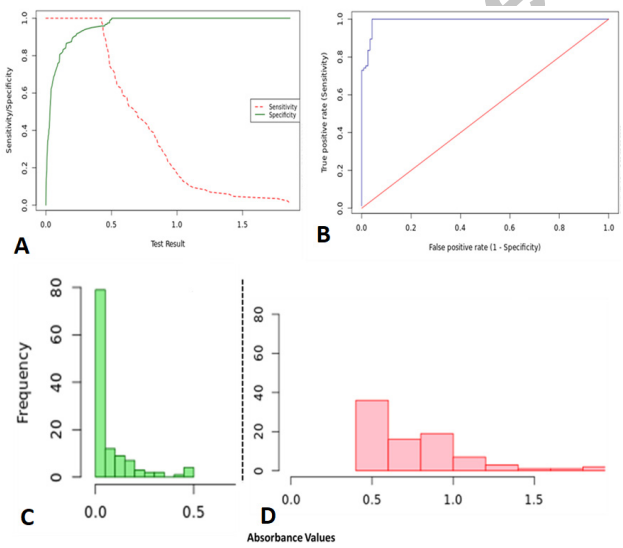

Fig. 2. (A) Inverse relationship between specificity and sensitivity and (B) Direct relationship between 1-specificty and sensitivity. The relationship between frequency and absorbance values (C) shows negative controls absorbance values ranges between 0 and 0.5. (D) and positive controls absorbance values are between 0.5 and 1.5.

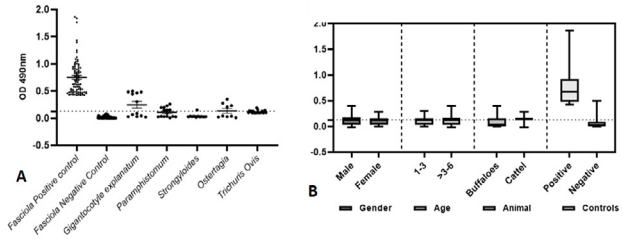

Fig. 3. **(A)** The scatter plot of OD values for other helminth parasites, including *Fasciola* positive and negative controls. **(B)** The box plot of OD between host sex, age and type. The positive control shows highest OD value, the cutoff point is set at 0.13.

DISCUSSION

Fascioliasis is a major veterinary disease all over the world, causing incalculable economic losses in livestock (Menkir *et al*., 2007). Faeces and liver samples examination recorded a prevalence rate of 28.4-78.0% in tropical countries (Keyyu *et al*., 2006). Globally, morphological traits and molecular approaches are utilized to distinguish adult trematodes species. The present study shows that the morphological parameters can be used for the differentiation of *Fasciola* specimens. External morphometric factors, particularly body size and shape, are among these indices. Other researchers have provided useful morphometric descriptions for distinguishing both species (Rouhani *et al*., 2017).

The current morphometrical analysis showed variations between the *Fasciola* species obtained from Pakistan with pure standards of *F. hepatica* from Iran and *F. gigantica* from Egypt. Considering the measurement and changes observed within the parameters like BL, BW and VS-P in the studied samples, they are likely to be grouped into *F. gigantica* like species. Because historically it has been suggested that *F. gigantica* might originate and spread by zebu cattle (*Bos indicus*) and water buffalo (*Bubalus bubalis*) in the Indian subcontinent (Amer *et al*., 2016). These results are in correspondence with the studies conducted by Chaudhry *et al*. (2020) in Punjab Pakistan. The measurement results agree with *Fasciola* species identified by Shafiei *et al*. (2014) in Iran, and Mufti *et al*. (2011) in Pakistan. In addition, previous studies showed that the incidence of *F. gigantica* is predominant in Punjab Province (Khan *et al*., 2009), just like in northern Iran (Ashrafi *et al*., 2004) due to its tropical and humid rainy climatic conditions where a large livestock industry exists. As most of infection with *F. gigantica,* found more commonly in tropical and subtropical regions of the world (Mas-Coma *et al*., 2014). BL, BW, VS-P, indices, and BL/

BW ratios were used to identify species in the current investigation, as proposed by prior studies (Lotfy *et al*., 2002). *Fasciola* in this study was found to be like *Fasciola gigantica* from Iran, India, and Egypt, as it shared the common phylogenetic origin and due to the movement of infected animals across the neighboring countries (Periago *et al*., 2006). *F. gigantica* has also been found in India and Mauritania (Raina *et al*., 2015). They have a lot in common with those from Thailand (Srimuzipo *et al*., 2000). The size of *Fasciola* adults varies according on the definitive host (Lotfy *et al*., 2002). *F. gigantica* in Pakistan is smaller than *F. gigantica* in Iran (Shafiei *et al*., 2014) due to the differences in geographical, ecological and other factors effecting parasite morphology. The variations in body length and width of the *F. gigantica* between the current and previous efforts could be impacted by geography. Despites other factors, the fixing and mounting of the specimens can be compromised too that may also have an impact on some metrics. In the earlier surveys besides, the fixation of single worm between glass slides or between a glass slide and cover slip as against the use of a relaxant in the other studies may have also unnaturally overstretched or distended the flukes (Srimuzipo *et al*., 2000).

The rDNA ITS-1 and ITS-2 have been used successfully to make an accurate diagnosis (Kostadinova *et al*., 2003). ITS-1 sequences have been utilized more frequently than any other marker for molecular identification of flukes. Its sequences are extremely reproducible making it very useful in molecular investigations. Although the ITS-I region is considered highly variable for higher-order phylogenetic analysis, the literature suggests that the 3′ end of the ITS-I region is a suitable marker for phylogenetic analysis for all members of the digenetic trematode genus, particularly the *Fasciola* genus, because this region is highly conserved within species (Hossain *et al*., 2011). The present study confirmed the existence of two *F. gigantica* genotypes (accession no. OM212803; OM212804) varied from each other at 15 base positions. Identified *F. gigantica* ITSI region exhibited close phylogenetic similarities to Chinese and Indian flukes, implying that they may have shared a common phylogenetic relationship. The present *F. gigantica* ITS-1 showed remarkable similarity 96-100% with *F. gigantica* ITS1 region from Iran, Vitenam and Kenya. On the other hand, the variation was seen in *F. gigantica* from Egypt (KF425321) at thirteen distinct nucleotide positions. However, there are hybrid and/or introgressed liver flukes that contain genetic material from both species in ruminants from Vietnam (Seid and Melese, 2018). The findings revealed a high degree of variation in present *F. gigantica* ITSI region are due to transversion and transition at multiple sites, which could be related to evolutionary pressure and aid in fluke evolution in geographically isolated regions.

The phylogeny backs up the BLAST results (Holder and Lewis, 2003). The current phylogenetic analysis of the ITSI region of *F. gigantica* shows that they belong to a clade with flukes from neighboring nations, which could explain parasite transmission by infected hosts moving between countries. These resemblances also suggest that they may have had a common evolutionary history. Buffalo and cattle fasciolids had the same base pairs and similar sequences in this study. The Pakistani *Fasciola* has BLAST results that are like *Fasciola* from other geographical isolates, indicating that it is related to *F. gigantica*. Molecular approaches have validated this from Japan, Korea, China, Spain, India, and Turkey based on their ITS-1 sequences distinguishes *F. hepatica* and *F. gigantica* (Alasaad *et al*., 2007). Investigations on ITS-1 and mitochondrial NDI gene sequences in Korea were made to differentiate aspermic *Fasciola* spp. (Rouhani *et al*., 2017). These flukes were divided into three haplotypes (Kor1, Kor2, and Kor1/2) based on ITS-1 rDNA, with similar nucleotides to *F. hepatica, F. gigantica,* and intermediate form, respectively.

Several investigations have been conducted on detection of coproantigen in feces for several helminthes (Lagatie *et al*., 2020). High level of sensitivity is an essential characteristic of ELISA based assays. In the present study the area under curve values was 0.991 (95% CI: 0.98-0.99; P<0.0001) which showed diagnostic accuracy of test 89.22% with 100% sensitivity and 82% specificity consistent with previous studies (Kowalczyk *et al*., 2018).

In present investigation, the overall prevalence of fasciolosis was found 42.7% with more infection in cattle as compared to buffaloes, consistent with previous study (Mufti *et al*., 2011). Different factors such as seasons, precipitation, and soil's structure are responsible for variable disease prevalence across different regions. However, higher humidity and increased population of snails may result higher prevalence of fasciolosis (Cruz-Mendoza *et al*., 2011). Significantly higher infection in females 24.6% compared to males 18.0% was obtained, consistent with Phiri *et al*. (2007). The reason could be higher stress and hormonal influence culminating in immune suppression and increased infection (Rezaul *et al*., 2015). Age-wise result showed the highest infection in >3-6 years as compared to 1-3 years age group. Similarly, it was recorded that the infection risk varies depending on the age factor as younger animals are less vulnerable to fasciolosis as compared to advanced age cattle (Mufti *et al*., 2011; Rezaul *et al*., 2015). This might be the result of higher parasitic access to older animals during grazing as youngers are not allowed to go out for grazing (Mufti *et al*., 2011).

CONCLUSION

In conclusion, the morphological and molecular investigations based on the ITS-1 sequences showed *Fasciola* seen in the Punjab area is like *F. gigantica* found in China and India. Overall prevalence of fasciolosis was 42.7% with coproantigen ELISA. The assay showed a diagnostic potential of 89.22% and was found suitable for our local setting to find the prevalence of suspected fasciolosis among livestock.

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IRB approval

The study protocol was approved No: BEC-FBS-QAU2017 by Bio Ethical Committee of Quaid-i-Azam University- Islamabad, Pakistan.

Ethical statement

The animals included in the study were slaughtered for other purposes to fulfill the protein demand of the population.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220416180444

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Molecular Identification and Prevalence of *Fasciola gigantica* in Cattle and Buffaloes of Punjab, Pakistan

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Supplementary Table I. Comparison of the inter specific variations between ITS-1 sequences of *Fasciola* spp. from Pakistan with nine reference *Fasciola* sequences obtained from GenBank.

Species	Country	Accession No.	Variable positions																			
			2	3	4	5	51	57	63	64	65	66	67	79	99	112	120	134	138	147	228	250	**391**
Fasciola A	Pakistan	OM212803	T	G	A	A	G	T	A	C	C	C	A	C	G	A	T	A	C	C	T	T	G
Fasciola sp.	China	AJ628426	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. gigantica	Iran	JN828953	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. gigantica	India	EF198867	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. gigantica	China	JF496711	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. gigantica	Egypt	KF425321	G	.	G	G	.	.	.	T	G	G	C	T	.	.	A	C	A	G	.	.	A
F. hepatica	China	AJ628432	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. gigantica	Kenya	KP760871	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
F. gigantica	Iran	MN784626	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
F. gigantica	China	AJ628425	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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0030-9923/2024/0001-0001 $ 9.00/0

Supplementary Table II. Estimates of evolutionary divergence between sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. This analysis involved 14 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 6794 positions in the final dataset.

***Fasciola* species accession no. country**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
F. hepatica AJ628432 China	0.00													
Fasciola sp. AJ628427 China	0.00													
F. gigantica AJ628425 China	0.01	0.00												
Fasciola sp. AJ628426 China	0.00	0.00	0.00											
Paramphistomum cervi MW567217 Pakistan	3.39	3.45	3.07	3.45										
F. gigantica MN970009 Vietnam	2.30	2.30	2.16	2.30	5.46									
F. gigantica MN784632 Iran	4.57	4.65	4.62	4.65	4.95	4.80								
F. gigantica KF425321 Egypt	5.77	5.80	5.80	5.80	3.51	5.29	5.07							
F. gigantica JN828953 Iran	2.42	2.31	2.29	2.31	4.42	4.93	3.23	4.82						
F. gigantica JF496711 China	3.63	3.65	3.63	3.65	4.25	4.16	3.53	4.49	5.40					
F. gigantica EF198867 India	2.66	2.76	2.66	2.76	4.25	3.56	3.61	4.25	3.23	3.08				
F. gigantica MN784626 Iran	4.57	4.65	4.62	4.65	4.95	4.75	0.01	5.03	3.15	3.44	3.61			
Fasciola gigantica isolate A (OM212803)	1.47	1.39	1.37	1.39	5.17	3.81	5.58	5.10	5.61	4.84	5.56	5.58		
Fasciola gigantica isolate B (OM212804)	3.83	3.96	4.02	3.96	3.92	5.79	5.71	5.24	5.80	5.18	5.93	5.71	0.66	0.00

Supplementary Table III. The prevalence and absorbance values of fasciolosis among host type, sex, and age groups from Punjab, Pakistan.

Characteristics		**No. of animals examined**	**Positive n (%)**	**x^2 P-vales**	**OD values (Mean ± SD)**	**95% CI (lower-upper)**
Host	Buffaloes	249	87(17.4)	12.18; p<0.001	0.09±0.09	0.08-0.10
	Cattel	250	126(25.3)		0.13±0.06	0.12-0.14
Sex	Female	317	123 (24.6)	5.36; p=0.02	0.10±0.08	0.09-0.11
	Male	182	90 (18.0)		0.12±0.09	0.10-0.13
Age	1-3	160	62 (12.4)	1.49; p=0.22	0.10±0.07	0.09-0.11
	>3-6	339	151(30.3)		0.12±0.08	0.10-0.12