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Morphological and Molecular Characterization of Rumen Fluke Species from Sheep in Southeastern Pakistan

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ABSTRACT

The present study was conducted to determine the prevalence of rumen fluke (Paramphistome spp.) infection in sheep slaughtered at the abattoir of Hyderabad, Pakistan and their molecular characterization with analysis of their evolutionary relationship. A total of 200 rumens from slaughtered sheep were examined and out of 200 sheep, a total of 75 (37.5%) were positive for rumen fluke infection. The results indicate that sheep were infected with the morphologically identical species indicating the only species infecting rumen. Further, it was found that the infection was prevalent in all months sampled but the highest infection rate was observed in November (56%) followed by October (38%), September (34%) and was observed lowest in August (22%). While, the infection rate in both sexes varies but the statistically non-significant difference in the prevalence among females (45%) than males (29%) was observed. Among the isolated rumen flukes, six flukes were used for molecular characterization by amplifying second internal transcribed spacer sequence flanking 5.8S and 28S ribosomal gene sequences (ITS-2+) yielding a 428 bp fragment which were sequenced for further analysis. The nucleotide sequences from 6 isolates were further analyzed for sequence similarities and were used for constructing phylogenetic tree. The sequence and evolutionary relationship analysis indicate that all isolates were belong to the same species and were clustered together with *Paramphistomum epiclitum*. Taken together, this study provides the baseline data for the prevalence of rumen fluke infection in southeastern Pakistan and their molecular characterization using rDNA ITS-2+ region which serve as useful genetic marker for species identification.

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Authors' Contribution
MR collected samples, performed the experiment, analyzed the data and wrote the manuscript. AGR, MBB and RSB designed the study, supervised the experiments and helped in manuscript writing. JAG, MN and ZAL helped in conducting experiments, data analysis and manuscript writing.

Key words
Prevalence, Rumen fluke, *Paramphistomum*, Sheep, ITS-2+, Pakistan.

INTRODUCTION

Amphistomiasis or paramphistomiasis caused by a trematode parasites of the genus *Paramphistomum* (rumen fluke) that infect a variety of ruminants with a global geographic distribution specially in tropical and subtropical regions (Horak, 1971; Diaz *et al*., 2006; Taylor *et al*., 2007). Animals become infected upon ingestion of viable metacercariae which excyst in the upper parts of the small intestine and actively penetrate the intestinal wall and enter the rumen where these flukes reside and attain sexual maturity (Hanna *et al*., 1988). The immature flukes are plug feeders in the intestine and cause hemorrhage, resulting into anemia, loss of weight gain and decreased production (Soulsby, 1982; Singh *et al*., 1984). Rumen flukes shares similarities in their life cycle and mode of infection with other trematodes but mature fluke resides in the rumen of the definitive host and results in non-symptomatic infection (Sarmah *et al*., 2014).

The geographical distribution of the rumen flukes is strongly associated with environmental conditions specially availability of water and wetlands. These conditions further support the survival of intermediate host and development and transmission of flukes (Tariq *et al*., 2008). In ruminants, amphistomiasis results in chronic infection which is most often sub-clinical, and therefore animals are often left untreated (Singh *et al*., 1984; Rolfe *et al*., 1994). Further, the lack of available techniques to detect immature flukes which results in severe damages are also major health concern. Due to damages caused by these flukes, disease causes considerable economic losses to the livestock industry, due to reduced productivity by induced hemorrhages and irregular rumination (Singh *et al*., 1984).

Despite the veterinary importance of rumen flukes, previous studies have mainly focused on morphology, life history, and epidemiology. Recently, rapid development of molecular techniques, especially the polymerase chain

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reaction (PCR), have provided valuable supplementary tools for the differential identification of digenean infection to overcome limitations of current morphological-diagnostic methods (Hong *et al*., 2013). Structurally, nuclear ribosomal DNAs (rDNAs) in eukaryotes are organized into tandem repeats. Each repeat has a transcriptional unit containing three genes (18S, 5.8S, and 28S rRNA) with two internal transcribed spacers (ITS-1 and ITS-2) separating these genes and an intergenic spacer (IGS) between the transcriptional units, different rDNA regions evolved differently. The ITS rDNA sequences provide useful genetic markers for parasite identification (Lofty *et al*., 2010). The intergenic spacer (IGS) rDNA possess considerable intra- and interspecific variation among parasites through repetition of sequences. However, IGS region of parasites is poorly characterized. The complete mitochondrial (mt) DNA sequence and the ITS-2 rDNA sequence of Paramphistomes were determined which can serve as an ideal target due to their high nucleotide substitution rates. Partial or complete mitochondrial genomes of parasitic flatworms have become very popular markers for detecting their presence in animals and for investigating their phylogenetic relationships at different levels (Itagaki *et al*., 2003; Lotfy *et al*., 2010). Along with the Paramphistosomes, ITS-2 also could be useful as a genetic marker for the molecular identification of *Fasciola hepatica*, *Dicrocoelium dendriticum* (liver flukes), and *Calicophoron daubneyi* (rumen fluke) in definitive and intermediate hosts. The secondary structure of the ITS-2 rDNA transcript (pre-rRNA) could provide information for identifying homologous nucleotide characters useful for cladistics inference of relationship. Such data could become taxonomic characters that can serve as a conserved marker (Krüger and Gargas, 2008).

To the best of our knowledge, molecular characterization of *Paramphistomum* spp. has not been described in Pakistan. Therefore, this study is proposed to perform the identification and evaluation of phylogenetic relationship of Paramphistomes recovered from sheep using amplification of ITS-2 region sequencing. Moreover, by concurrently assembling locality and host data, a database can be constructed that will be used to evaluate Paramphistome host–parasite associations and biogeography. Such efforts will also culminate in better means for identification of Paramphistome in sheep, and the complete understanding of their phylogenetic relationships. The ultimate outcome of the present study is to provide the baseline information that will help in the control of such parasites in animals and humans.

MATERIALS AND METHODS

Sample collection

Adult rumen flukes were collected from the rumen of the slaughtered cross breed sheep at central abattoir of Hyderabad, Pakistan. The demographic data of infected animals were recorded including age and sex. The specimens of rumen flukes were preserved in plastic bottles containing 70% ethyl alcohol and brought to the Molecular Parasitology Laboratory, Sindh Agriculture University, Tando Jam Pakistan.

Identification of parasites

The collected rumen flukes were identified through conventional method as described by Singh and Srivastava (1977).

Parasite fixation

The adult worms were washed in water and placed on a glass slide, while another glass slide was placed to cover them individually. The specimens were left in 10% formalin for 24 hours and then removed and washed in distilled water for 3 days.

Parasite staining

Rumen fluke were stained using Borax carmine stain. Briefly, 2 grams of Borax Carmine were dissolved in 50 ml of distilled water and heated in a water at below boiling level in water bath for 60 min than it was allowed to cool. The 50 ml of absolute ethyl alcohol was mixed in Borax Carmine solution. Each specimen was shaken in the staining solution for 4 days. The specimens were then removed from the stain and de-stained in 1% hydrochloric acid for 24 hours. The acid was thoroughly washed out from the specimen using tape water. The specimens were then dehydrated through a series of dilutions including 50, 70, 90, 95 and 100% alcohol for 1 hour each, and cleared by using xylene for 30 min.

Smear examination

The cleared specimens were placed on glass slides while a drop of Canada balsam was added to each glass slide and then covered with a cover slip. The mounted slides were allowed to air dry and viewed under the stereoscopic microscope for identification of the flukes. The identification of parasites was done with the help of morphological keys.

Genomic DNA extraction

Before extraction of genomic DNA, the samples were thoroughly washed in distilled water to remove remaining ethanol. The genomic DNA from flukes and blood samples was extracted using commercial kit (DNeasy Blood and Tissue Kit #69504 Qiagen, Germany). Briefly, 180 µl of buffer ATL was added to the 200 µl of whole blood or homogenized parasite and mixed thoroughly by vertexing

to obtain a uniform suspension. After homogenization, 20 µl Proteinase-K was added and mixed immediately in 1.5 ml collection tube. The suspension was then incubated at 56°C until tissues are completely lysed. Afterwards, 200 µl of AL buffer was added and incubated for 10 min at 70°C and then 200 µl of absolute ethanol was added and mixed immediately by vertexing. The suspension was then transferred to a Genomic DNA extraction column inserted in a collection tube and centrifuged at 6000 × *g* for 1 min. The tube containing flow-through was discarded and the column was washed with 500 µl wash buffer AW1 and centrifuged at 6000 × *g* for 1 min. The flow through was discarded and column was washed with 500 µl wash buffer 2 AW2 and centrifuged at 20000 × *g* for 2 min. The column was then dried using an additional centrifugation at 20000 × *g* for 3 min. Finally, genomic DNA was eluted using 100 µl of elution buffer (AE buffer) by incubating for 2 min at room temperature followed by centrifugation at 6000 × *g* for 1 min. The purified genomic DNA was quantified using Nano Drop™ 1000 spectrophotometer (Thermo Scientific, USA) and immediately used or stored at –20°C until further processing.

PCR amplification and sequence analysis

The ITS-2 rDNA region including the flanking 5.8S and 28S sequences (ITS-2) were amplified by PCR using already reported primers; ITS-3S (forward): (5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and A28 (reverse): (5'-GGGATCCTGGTT AGTTTCTTTTCCTCCGC-3'), as described by Goswami *et al*. (2009). The PCR amplifications were performed using reaction mixture contained: 12.5 µl Master Mix, 25pmol of each primer, 50 ng of DNA template and the final volume of 25 µl PCR reaction mixture was adjusted by adding nuclease free water. PCR amplification was carried out in thermal cycler (Applied Biosystem 2720, USA) under following conditions: initial denaturation for 4 min at 94°C followed by 36 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing and extension for 1 min at 72°C. Final extension was carried out at 72°C for 10 min (Goswami *et al*., 2009). PCR products were then run on Agarose gel and amplified fragments were then sequenced for further analysis of their sequence variation and phylogenetic analysis.

RESULTS

The present study was carried out to record the prevalence of Paramphistomiasis in sheep slaughtered at small ruminant abattoir in Hyderabad, Pakistan. A total of 200 rumens of freshly slaughtered sheep were examined to record the prevalence of Paramphistomiasis.

For identification of rumen flukes using conventional morphological keys, the adult stomach flukes collected from the rumen were carefully examined and differentiated because of less variation in size and morphology between different species. Rumen flukes were preliminarily identified under microscope using low power magnification and then slides were prepared for identification and detailed morphological studies. The collected flukes were placed on Petri dish and observed through stereo microscope to clarify the morphology. Final identification of *Paramphistomum* spp. was done based on morphological characteristics of flukes; its shape, anterior sucker, posterior sucker (acetabulum) following the standard guidelines given by Urquhart *et al*. (2008). Parasites were processed for whole mounting and stained by Borax carmine according to the procedure given by Singh and Srivastava (1977). In the present study, most of the species were of related to *Paramphistomum epiclitum*, which were found mainly in the rumen of sheep and goat and were light pink in color with a sucker at the tip of the cone and another sucker ventrally at the posterior end (Supplementary Fig. 1). The body of isolates were elongated with blunt ends and pear-shaped, slightly concave ventrally (conical) and convex dorsally. The worm measures about 8-8.9 mm in length and 2.71-3.0 mm in width at anterior end of posterior third or at its junction with middle third. Mouth was terminal, funnel-shaped, widened posteriorly. Acetabulum measured as 1.62-1.75 mm in diameter.

To record the prevalence status of Paramphistomiasis, 200 rumens of fresh slaughtered sheep were examined for presence of Paramphistomes and out of them 75 (37.5%) were found infected with *Paramphistomum* spp. Further, data revealed that during the study period (August to November), overall prevalence was higher in cool weather as compared to temperate months as observed 22% (11), 34% (17), 38% (19) and 56% (28) in month of August, September, October and November, respectively (Table I). While the month wise frequency of infection was recorded as 14.66%, 22.66%, 25.33% and 37.33%, respectively. The degree of prevalence among these months was statistically significant at $p < 0.05$.

Moreover, gender wise prevalence of rumen flukes was also recorded during the study period to observe risk/exposure factors involved in the abundance of infection in studied locality. Overall prevalence recorded showed that out of 100 males, a total of 29 (29%) were infected whereas 45 (45%) out of 100 infected were female sheep (Table II). The frequency of male infection was recorded as 13.7, 24.1, 27.5 and 34.4% in the month of August, September, October and November. While the frequency rate of infection in females during these four months were recorded as 15.5, 22.2, 24.4 and 37.7%, respectively. The infection rate in

Table I. Prevalence of *Paramphistomum epiclitum* in sheep at Hyderabad.

S.No	Month	No. of animals examined	No. of animals +ve	Prevalence (%)	Frequency (%)
1	August	50	11	22	14.666
2	September	50	17	34	22.666
3	October	50	19	38	25.333
4	November	50	28	56	37.333
Total		200	75	37.5	100

Chi sq.: 7.933, *p* value: 0.0474, df: 3, Significant at $p< 0.05$.

Table II. Prevalence frequency of Paramphmistomiasis in male and female sheep at Hyderabad.

S. No	Month	Male				Female			
		Exam.	Posit.	Preva. %	Freq.%	Exam.	Posit.	Preva. %	Freq.%
1	August	25	4	16	13.7	25	7	28	15.5
2	September	25	7	28	24.1	25	10	40	22.2
3	October	25	8	32	27.5	25	11	44	24.4
4	November	25	10	40	34.4	25	17	68	37.7
Total		100	29	29	100	100	45	45	100

Chi Sq: 3.459, *p* value: 0.0692, df: 1.

both sexes varies but degree of prevalence among male and female was statistically non-significant at $p>0.05$.

Due to limitations of the conventional methods to identify and differentiate the similar species within one group, a method needs to be developed to specifically identify and differentiate related species. It is very difficult to identify and differentiate the species of amphistomes on the basis of its morphology due to significantly less variation in size and shape of parasite and its internal organs, which varies even in mature and immature worms of same species. However, morphology cannot be very well appreciated in the stereo microscope (Choudhary *et al*., 2015). This is particularly the case for soft-bodied animals such as digenean trematodes. PCR-based techniques targeting the rDNA ITS2 sequences, which is located between the 5.8S and 28S coding regions, have proven to be a reliable tool to identify the helminth species and their phylogenetic relationships (Blair *et al*., 1999). The second internal transcribed spacer (ITS-2) sequences in nuclear ribosomal DNA, which is located between the 5.8S and 28S coding regions, have proven useful for diagnostic purposes at the level of species (Adlard *et al*., 1993).

For molecular identification, genomic DNA was extracted from 6 rumen flukes and concentration was measured by spectrophotometry. The DNA concentration of samples ranged from 24.8 to 69.1 ng/µl at light absorbance of 230 nm level and purity at 260/280 nm wavelength, ranged from 1.70 to 1.86. The extracted total genomic DNA contained DNA from Paramphistome, host species and any biological contamination. The PCR analyses of ITS-2 region amplification from all the specimens produced identical fragments with a length of about 500 bp (Supplementary Fig. 2). The sequence analysis of the ITS-2 region of rDNA from each isolates specimen of this study showed that all fragments are of 428 bp in length and no intra-specific variation was observed in the nucleotide composition of the ITS-2 region (homology = 100%) (Fig. 1).

The isolates obtained from sheep in Hyderabad region were further analyzed for accuracy in the identification and their evolutionary relationship. The specific identification of parasites was done by PCR amplification of gDNA was further confirmed by sequencing of amplified fragments. For confirmation of species sequences, all ITS-2 PCR products were subjected to direct sequencing and sequences were aligned by the Clustal W. All 6 isolates showed similar nucleotide sequences. While BLAST hit results shows that the query ITS-2 sequences were more similar (99%) to the sequences of *Paramphistomum epiclitum* (NCBI Accession No. JX678260, KF642983). The BLAST results revealed that the isolate of present study is recognized as *Paramphistomum epiclitum*. The phylogenetic tree based on ITS-2 region of rDNA sequence of *Paramphistomum* isolates was constructed. For constructing phylogenetic tree, initially sequences were aligned using Clustal W multiple alignment tool with the

Isolates	Sites of Bases																																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Amphi-HYD-1	C	T	T	A	T	A	A	A	C	T	A	T	C	A	C	G	A	C	G	C	C	C	A	A	A	A	A	G	T	C	G	T	G	G	C	T	T	G
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77
Amphi-HYD-1	A	A	T	C	T	G	C	C	A	G	C	T	G	G	C	G	T	G	A	T	T	T	C	C	T	C	T	G	T	G	G	T	T	T	G	C	C	A
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116
Amphi-HYD-1	G	T	G	A	G	G	T	G	C	C	A	G	A	T	C	T	A	T	G	G	C	G	T	T	T	T	C	C	T	A	A	T	G	T	C	T	C	C
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155
Amphi-HYD-1	G	A	C	A	C	A	A	C	T	G	C	G	T	C	T	T	G	C	T	G	G	T	A	G	C	G	C	A	A	A	C	G	A	G	G	G	T	G
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194
Amphi-HYD-1	G	G	C	G	G	T	A	G	A	G	T	C	G	T	G	G	C	T	C	A	G	T	G	A	A	C	T	G	T	A	A	T	G	G	T	A	G	C
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233
Amphi-HYD-1	C	G	C	T	C	T	A	T	T	G	T	T	G	T	G	C	C	T	T	T	G	T	T	A	G	T	G	C	A	A	C	T	G	G	T	T	T	G
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272
Amphi-HYD-1	G	A	T	G	C	T	A	T	T	G	C	T	G	T	C	C	G	T	C	C	A	A	T	G	A	C	G	A	T	C	A	C	C	T	A	C	T	G
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	274	275	276	277	278	279	280	281	282	283	284	285	286	287																								
Amphi-HYD-1	G	G	T	G	T	T	C	T	A	T	T	A	C	C																								
Amphi-HYD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.																								
Amphi-HYD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.																								
Amphi-HYD-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.																								
Amphi-HYD-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.																								
Amphi-HYD-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.																								

Fig. 1. Sequence alignments of ITS2 sequences of isolate of present study. Base homologies are indicated with dots (.), gaps are indicated with hyphens (-), and base changes are indicated with the substituted base.

default gap and extension penalties. ITS-2 sequences were entered in the Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 for construction of the phylogenetic trees using maximum parsimony and distance methods namely the Neighbor-Joining, UPGMA and Minimum Evolution. Branch support was given using 1000 bootstrap

replicates. The optimal tree with the sum of branch length = 0.18470714 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 240 positions in the final dataset. The phylogenetic analysis of ITS-2 region of rDNA of *Paramphistomum* isolates of the present study revealed the sequences of analyzed strains segregated into four main groups. Whereas, isolates of the present study were closely related to *Paramphistumum epiclitum* (Fig. 2). Sequences from all 6 isolates of this study were not shown variation and these isolates were distantly related to sequences of *Calicophoron shillogensis* (JX678251). Further, the multiple sequence alignments show conservation in the sequences of our isolates (AMPHI-HYD) and other related species within the same group (Fig. 3).

DISCUSSION

Paramphistomosis causes severe economic losses to ruminant production by reducing milk, meat and wool production since the flukes sap nutrients from their hosts and severe damages caused by immature flukes during migration. This study reported the current status of *Paramphistomum* infections in sheep and its molecular characterization in southeastern Pakistan.

Along with coprological observations, the examination of rumen to assess the prevalence of adult flukes in a group of animals presented for slaughter is also an effective way of diagnosis of rumen fluke infection. In the abattoir study, determination of potential risk factors that predispose animals to acquire an infection are complicated by lack of independence between explanatory variables. However, determining effects and potential interaction with risk factors for acquiring infection, a much larger abattoir study would be needed.

The results of present study have revealed the endemicity of *Paramphistomum* infection in studied area with a prevalence rate of 37.5% in the rumen of slaughtered sheep. Th only rumen fluke species detected in the present study was *Paramphistomum epiclitum*. This species has previously been reported to be infecting sheep (Gicik *et al*., 2003). Although there are limitations to analyze the effect of seasonality on infection rate due to specified sampling timing but there is much fluctuations in temperature and rainfall which also considered as factors affecting prevalence of rumen fluke infection. During the study period it was found that overall higher prevalence was in late summer (November) with lowest in the monsoon season. Similarly, the infection rate of another species from the same genus of rumen flukes, *Paramphistomum daubneyi,* was decreased in July and August, while the sudden increase in the infection rate was observed postmonsoon during the months of September upto January (Mage *et al*., 2002; Galdhar and Roy, 2005). Moreover, it was observed that females possess higher infection rate when compared with males. Meanwhile, previous studies reported a significant difference between infection rate in both sexes with higher prevalence rate in males when compared with females (Khan *et al*., 2008). These differences may be attributed to the environmental factors such as grazing patterns and treatment activities.

Ruminant infection with the trematode species largely depends on the presence of intermediate hosts (aquatic snails) mainly belongs to the Lymnaeidae family, thus the areas with provision of excessive water or wet areas are at risk. The previous studies reporting the prevalence of rumen flukes throughout the year suggest that there is either lack of proper usage of ant-helminthic drugs or the presence of intermediate host snails over whole year (Abrous *et al*., 1999; Dreyfuss *et al*., 2008). However, in general the temperature dependent prevalence of fluke infections was recorded, being higher in summer with more abundance postmonsson and lower in winter months. It has been suggested that the mud snail, *Galba truncatula*, which serve as an intermediate host for fluke species including rumen and liver flukes (Gordon *et al*., 2013). This indicates that the same intermediate host may serve as a source of mixed fluke infection. Previous studies revealed that the decreased prevalence of rumen flukes in winter may be connected with the non-availability of intermediate snail host with serves as an infection source (Tariq *et al*., 2008). Further, the outbreaks of paramphistomosis generally reported in summer with drier months (Soulsby, 1982). However, lower abundance in early winter with increase in the prevalence rate during rainy season or postmonsoon was also detected in ruminants (Hassan *et al*., 2005). In agreement with the present findings, higher prevalence with clinical paramphistomosis has been reported in calves during late summer and early winter months (Rolfe *et al*., 1991).

Among the most prevalent helminth parasites, few trematode species seems to cause frequent infections in ruminants including sheep and cattle. The most prevalent trematode species belongs to the genera *Paramphistomum*, *Fasciola* and *Dicrocoelum* (Arias *et al*., 2011). However,

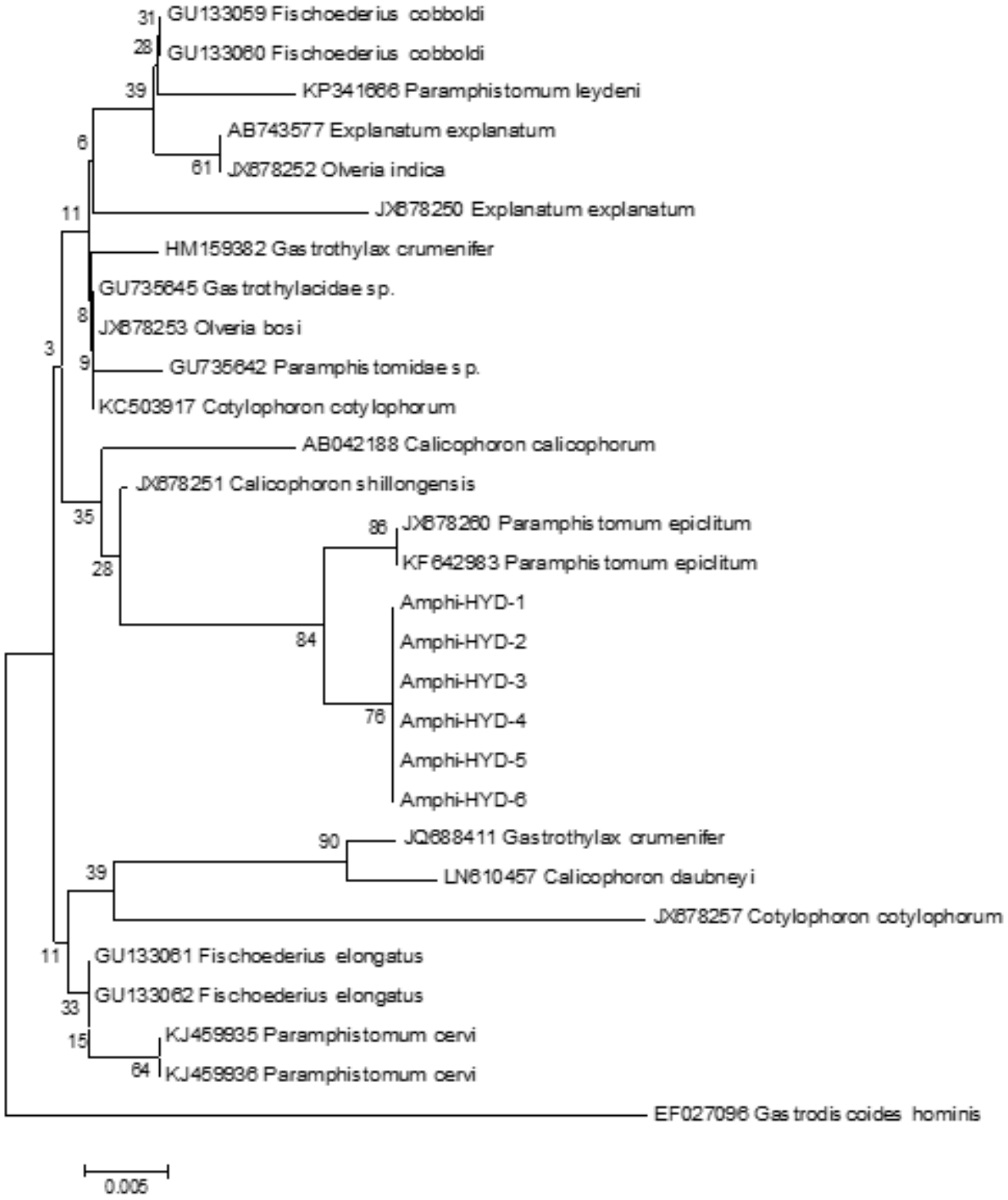

Fig. 2. The phylogenetic tree analysis of ITS2 region of rDNA sequences of different trematodes species. The rumen fluke isolates collected from native sheep of Hyderabad, Sindh province, Pakistan shows the isolates were closely related to *Paramphistumum epiclitum* and evolutionary conservation between different trematode species.

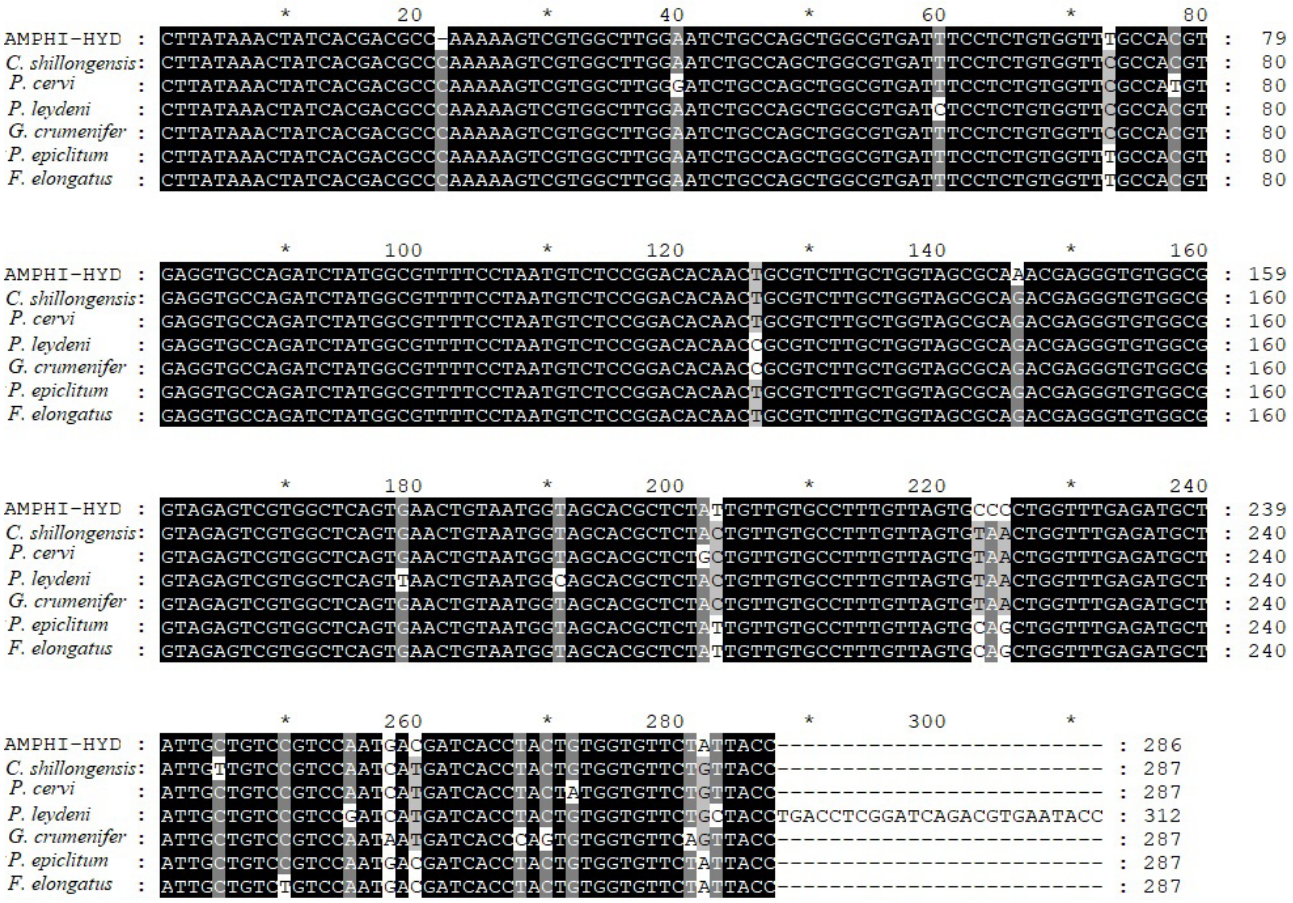

Fig. 3. The multiple sequence alignment of rumen fluke isolates identified in the current study (AMPHI-HYD) and other related species. (*Calicophoron shillongensis; Paramphistomum cervi; Paramphistomum leydeni; Gastrothylax crumenifer; Paramphistomum epiclitum; Fischoederius elongatus*).

rumen fluke (*Paramphistomum*) species cause significant economic losses but less studied as compared to other trematode species. Together with the present findings, data reveals that several species of the trematodes including rumen fluke were reported globally with the species like *P. epiclitum, P. cervi, P. leydeni, C. calicophorum, C. spatiosus, G. crumenifer, D. dendriticum, F. hepatica, F, gigentica* have been reported from several regions of Asia and Europe (Dorchies *et al*., 2002; Gordon *et al*., 2013; Jadav *et al*., 2018). However, the prevalence of rumen flukes varies greatly among different geographical areas, like in our study there was higher prevalence in the month of November as compared to rest of the months observed.

The similar finding also reported from India and France stating the higher prevalence in the beginning of winter with lower abundance in warmer months (Rolfe *et al*., 1991; Mage *et al*., 2002; Galdhar and Roy, 2005; Tariq *et al*., 2008).

Due to chronic infection with mild pathogenicity caused by the adult flukes, rumen fluke infection has been neglected but the severe infection can cause serious effects on the health of an animal and economic losses to the farmers. However, the accurate diagnosis is still important to identify and treat the infected animals in order to reduce the risk of economic losses. Currently, sequence analyses of the ribosomal DNA genes encoding for structural RNAs of ribosome have been widely used in the phylogenetic studies and identification of the trematodes, since ribosomal DNA is highly conserved with more variable regions. Along with rDNA genes, ITS region has been shown to exhibit length and sequence polymorphisms thus can be useful in discriminating both closely related species and population of a given species (Hillis and Dixon, 1991). Differences of even one nucleotide change in the ITS sequence can be used as an effective genetic marker to distinguish closely related digenean species. Current study focuses on the characterization of ITS-2 sequences of *Paramphistomum epiclitum* and were compared with the corresponding

sequences of other trematode species. When compared with the 18S rDNA which limits the use of this gene as a genetic marker due to slight sequence divergence, ITS-1 and ITS-2 sequences can be used as an important molecular tool to specifically discriminate different species within the same group. Based on current methodology and obtained data, we can distinguish *Paramphistomum epiclitum* from other trematode species which are morphologically closely related and the PCR amplification of ITS-2 region and its sequence analysis further provided an efficient tool for evolutionary relationship of different helminth species. Present findings revealed that all the isolates obtained from sheep in Hyderabad yields a fragment of 428 bp on agarose gel during PCR amplification and sequence analysis in the phylogenetic tree shows that they all were clustered together along with *Paramphistomum epiclitum* and within the same clade of another rumen fluke species, *Calicophoron* spp. While, another intestinal species (*Gastrodiscoides hominis*) belonging to the same family was used as an outgroup. However, due to limited available sequences identified from the same region it is bit complicated to draw an evolutionary relationship between different species. Taken together, data from the present study revealed that sheep present in the southeastern regions of the Pakistan are exposed to the infection by *Paramphistomum* spp. which indicates that there is dire need for control measures. While, efforts are required to control intermediate host by either controlling their habitats or deworming animals to reduce the risk of further persistence of infection.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190506070509

Conflict of interest

The authors have no conflict of interest.

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