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Molecular Characterization of *Paramphistomum cervi* in Buffaloes

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ABSTRACT

***Background*:** Paramphistomiasis (Rumen fluke disease) in ruminants is a major health problem, characterized by coarse hair, weakness, loss of appetite, weight retardations, intestine ulcers, inter-mandibular inflammation, causing substantial economic losses, and high mortality. In tropical and subtropical regions, the disease was neglected but has recently emerged as an important cause of production losses. While documented reports on *Paramphistomum cervi*, *Paramphistomum ichikawai* and *Paramphistomum* are limited in Asian countries and paramphistomosis has been considered the major health and economic problem in several countries. The present study aimed to identify paramphistomoid flukes that infects buffaloes with the goal of characterization of prevalence in Pakistan and its comparison with neighbor countries.

***Materials, Methods & Results*:** In 2018, a total of 178 slaughtered buffaloes aged four to six years were examined. After an immediate postmortem examination of each buffalo, flukes were collected from their infected rumen and reticulum using sterilized forceps and placed in a saline solution. DNA was extracted from adult *Paramphistome* species using the standard phenol chloroform method and used for amplification of partial fragment of 18S rRNA sequences using specific pair of primer. After amplification and sequencing of 18S rRNA partial fragment, the generated sequences were assembled and trimmed to remove any primer contaminations. Twenty-three randomly selected and morphologically identified adult *Paramphistomum* were used in species-level identification using specific primers for partial fragment of 18S rRNA sequences. The cleaned sequences (810 bp) were used to identify similar sequences using BLAST on the NCBI website. The GenBank retrieved sequences and new *Paramphistomum* species isolated sequences were aligned using CLUSTAL in the BioEdit Sequence Alignment Editor. In addition, a phylogenetic tree was constructed using maximum likelihood method in MEGA X. The 18S rRNA sequence was found 100% similar with *Paramphistomum cervi* of China and 98% with *Paramphistomum epiclitum* and other *Paramphistomum* species of India. The parasitic *Pharamphistomum* species was identified molecularly as *Paramphistomum cervi*.

***Discussion*:** Molecular studies provide insight into the biology and phylogenetic relationship among various parasites. These studies are reliable in the genetic-based identification and description of several disease causing agents. The 18S rRNA sequence of *Paramphistomum cervi* generated in this study was found closely identical to the *P. cervi* of the neighbor countries (China and India) which may be due to the similar geographical, environmental conditions and transboundary movement of infected hosts. This is the first nature of study which provides the molecular-based evidence of *P. cervi* existence in Pakistan and revealed the 18S rRNA as novel molecular marker for the identification and further characterization of *Paramphistomum* species across Pakistan. The submitted sequence of this study will provide a baseline for further molecular characterization and to compare with other *Paramphistoma* species from different regions of Pakistan.

Keywords: Paramphistomoid, *Paramphistomum* spp., molecular identification, 18S rRNA, Pakistan.

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INTRODUCTION

The Paramphistomidae family comprises numerous species of *Paramphistomes* (commonly known as rumen fluke) trematodes infecting rumen and reticulum of ruminants globally [19,7]. More than seventy species of the superfamily Paramphistomoidea have been identified in different geographical regions [1]. Ruminant's paramphistomiasis (rumen fluke disease) are due to the pathogenic activities of *Paramphistoma* species of the genus *Paramphistomum* [32]. The infection is characterized by coarse hair, weakness, loss of appetite, weight retardations, intestine ulcers, inter-mandibular inflammation, causing substantial economic losses, and high mortality [4]. In tropical and subtropical regions, the disease was neglected but has recently emerged as an important cause of production losses [28].

The prevalence of paramphistomosis is high throughout tropical and subtropical regions, specifically in Africa, Asia, Australia, Eastern Europe, and Russia [16]. Particularly in Asian countries, *Paramphistomum* infections due to *Paramphistomum cervi*, *Paramphistomum ichikawai* and *Paramphistomum microbothrium* remain widespread and in some areas 30-60% prevalence have been evident [23]. In Pakistan, approximately 56% contribution in agriculture is due to the sub-sector livestock [24]. The prevalence of gastrointestinal helminth has been evident infecting 25.1 to 92% of ruminants [12,14]. Among these, Paramphistomosis has been considered to be the major health and economic problem in several regions [9].

The paramphistomes are usually identified based on morphological and histological examinations, however, due to morphological similarities the accurate identification requires molecular approaches [25]. Comparative genomic studies through the sequencing of ribosomal genes are particularly used in diverse fields of research [15]. Molecular markers including mitochondrial DNA and 18S rRNA regions of the nuclear ribosomal DNA (rDNA) are frequently used in the identification of trematode species [21,22]. The parasitic species are prevalent in various regions of Pakistan however; little considerations have been taken in the utilization of molecular tools for their identification. Therefore, the present study was designed to provide molecular level identification using 18S rRNA sequence of *Paramphistomum* species and compare with other countries.

MATERIALS AND METHODS

Study area

The present study was conducted in four districts namely Mardan (34°11'54.6"N, 72°01'37.4"E), Swabi (34°7'12"N, 72°28'12"E), Charsadda (34°09'49.4"N, 71°44'53.4"E), and Nowshera (33°59'54"N, 71°59'54"E) located in the central part of KP province of Pakistan. Throughout the province precipitation is variable and the climate varies with elevation; an increase in temperature in the least elevated regions and decreases at high elevation with snowfall on the peak of mountains in northern regions [15].

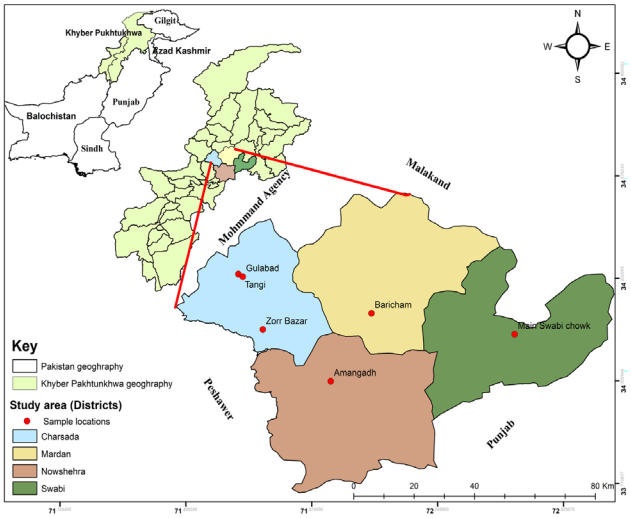

Figure 1. Map of the study area including sampling locations (red dots).

Sampling

In 2018 a total of 178 slaughtered buffaloes of four to six years of age were examined (Figure 1). After an immediate postmortem examination of each buffalo, flukes were carefully collected from the infected rumen and reticulum using sterilized forceps and placed in a saline solution (0.9% NaCl, 0.45% glucose) [32]. Some flukes were preserved in 70% ethyl alcohol. The flukes were then shifted to the parasitology laboratory of Abdul Wali Khan University Mardan for further analysis. Species identification was performed according to the previously published outlined criteria [5,8,34].

DNA extraction

DNA was extracted from adult *Paramphistome* species using the standard phenol chloroform method [26]. The collected adult *Paramphistomes*

were washed with PBS following pulverization and grinding with mortar and pestle in the presence lysis buffer (Tris HCl 10 mM pH 8.0, EDTA 100 mM, and SDS 10%). The grinded tissue samples were transferred into a 1.5 mL microtube and 5 µL of Proteinase k (20 mg/mL) was added into it. The samples were incubated at 37°C overnight. Following centrifugation at 13,000 *g* for 10 min an equal amount of phenol and mixture of chloroform (1:1) and isoamyl alcohol (24:1) was added to the sample and centrifuged at 13,000 *g* for 10 min. The pellet was discarded, and supernatant was transferred into a fresh microtube and again centrifuged by adding 500 µL of chloroform and isoamyl alcohol (24:1) at 13,000 *g* for 10 min. Again, the pellet was discarded, and DNA was precipitated from the supernatant by adding 40 µL of sodium acetate solution and 500 µL of isopropanol. The samples were centrifuged at 15,000 *g* for 10 min. Supernatant was discarded and the pellet was washed with 70% ethanol by centrifugation at 13,000 *g* for 15 min. After centrifugation, Ethanol was removed using cotton swab and samples were kept open at 37°C for air dry. In the last 100 µL of TE buffer was added into the microtubes containing DNA and were stored at -20°C for further processing [30]. The extracted DNA was stored at -20°C and were used for amplification of partial fragment of 18S rRNA sequences (810bp) using specific pair of primer; 18S forward 5'-TCT GTGATGA CTCTGGAT-3' and Reverse 5'-ACC ATTCAAT CGGTAGTA-3' described previously [27].

A 20 µL final reaction mixture containing 10 µL master mix (2x), 1 µL each forward and reverse primer, 1.5 µL template DNA, and 6.5 µL PCR water was prepared. For amplification the initial denaturation temperature was set on 95°C for 5 min followed by denaturation temperature 94.5°C for 30 s (35 cycles), annealing temperature 58°C for 30 s, extension temperature 72°C for 1 min, and final extension temperature 72°C for 5 min. The reaction was held on 4°C till further analysis. A standard 100 bp DNA ladder and the PCR products were analyzed by 2% agarose gel stained with 0.5 µg/mL ethidium bromide and visualized under the GeneDoc (UVP BioDoc-It Imaging System). Positive PCR products were submitted at Macrogen, South Korea for sequencing using the aforementioned primers.

Sequence analysis

The obtained sequences in the study were assembled in DNASTAR V5.0[1] and trimmed to remove any primer contaminations. The sequence was deposited to NCBI GenBank under accession number (MT707654). The purified sequence was BLAST in NCBI to scan for similar sequences deposited from different regions for the same gene fragment. The scanned *Paramphistomum* species sequences were downloaded and aligned in the BioEdit Sequence Alignment Editor v 7.0.5[2] and a phylogenetic tree was constructed using maximum likelihood method keeping 1000 bootstrap replicates for branch support in MEGA v X software[3]. A distantly related trematode species *Taenia solium* (Accession: Q260091) was taken as an outgroup to examine the reliability of the phylogenetic tree.

RESULTS

Twenty-three randomly selected and morphologically identified adult *Paramphistomum* were used in species-level identification using specific primers for partial fragment of 18S rRNA sequences of extracted genomic DNA through PCR. The sequence obtained in the study was found with the 49% A+T and 51% G+C nucleotide composition.

BLAST analysis of present study 18S rRNA sequence showed a 98%-100% similarity with *Paramphistomum* species from cattle of India (JX678271) and China (KJ459934, KJ459935, KJ459936, KJ459937, KJ459938). Based on 100% sequence similarity with *P. cervi* of China, the collected isolate was considered *P. cervi*. The evolutionary tree was constructed which revealed the similarity of the current study sequence with the *Paramphistomum* species of the neighbor country. The phylogenetic analysis of present study isolate forms a monophyletic clade with other *Paramphistomum* species including *P. cervi* and *P. epiclitum* and form a subclade with *P. epiclitum* (JX678226, JX678283) from India and *P. cervi* (KJ459934, KJ459935, KJ459936, KJ459937, KJ459938) from China. Separate subclade was made with *P. epiclitum* (JX678271, JX678244) from India, *P. cervi* (KT198987, KF475773) from China, *Paraphistomum* (AB688990) from Saudi Arabia, and *P. epiclitum* (LC113920, LC113923, LC113921, LC113924) from Thailand (Figure 2).

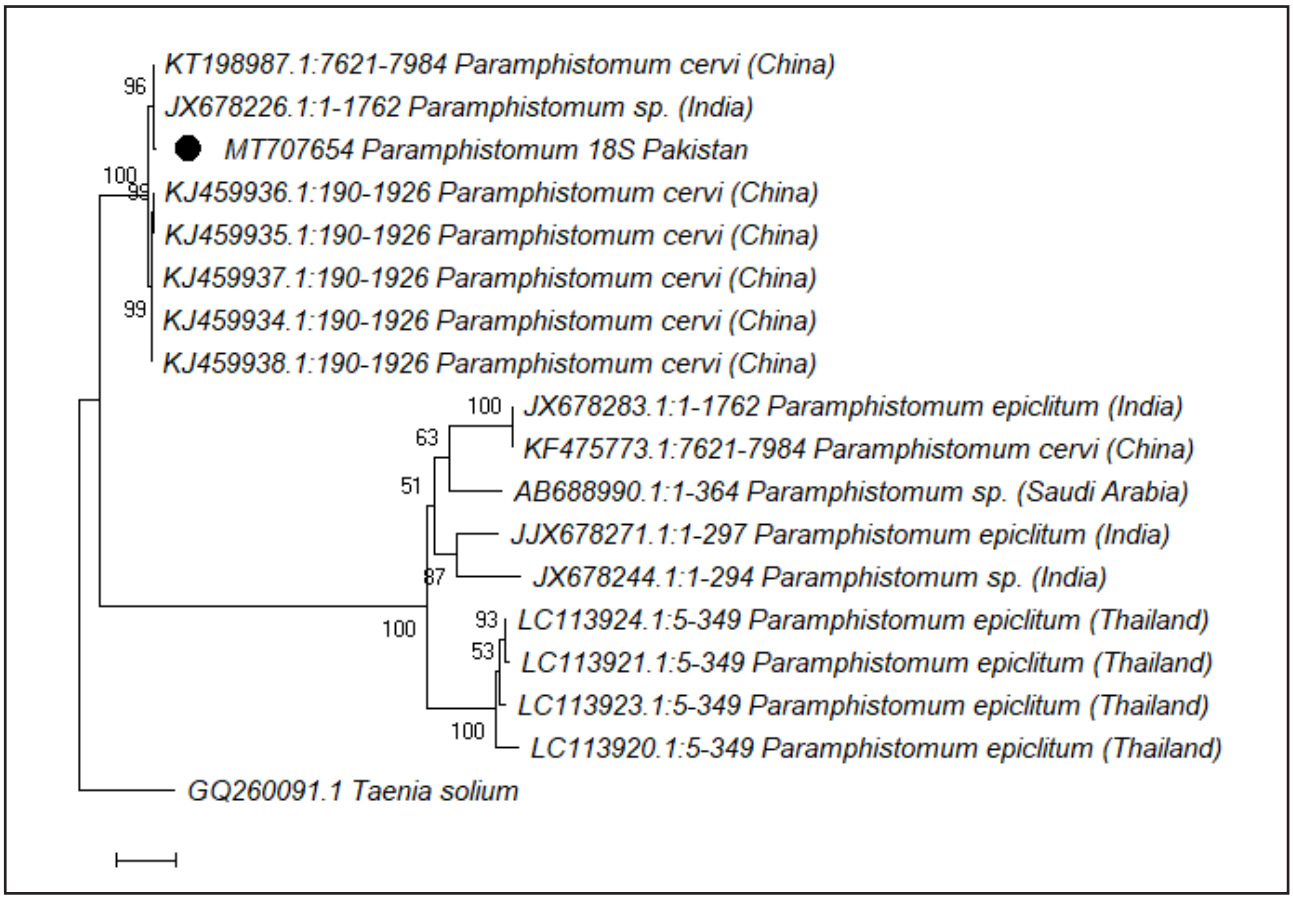

Figure 2. Maximum likelihood tree inferred from partial fragment of 18S rRNA sequences of *Paramphistomum* species using *Taenia solium* (Accession: GQ260091) as outgroup. GenBank accession numbers are followed by the species name and values (Bootstrapping values) that were indicated at each node. The bar represents 0.050 substitutions per site.

DISCUSSION

Paramphistomosis is widely prevalent under the influence of a multifactorial system including parasitic agents, hosts, transmission processes, and environmental effects [13]. The infection is due to several indistinguishable *Paramphistomum* species for which morphological and molecular techniques are used [25]. In the present study flukes were collected from the infected rumen and reticulum of buffalos of four to six years of age. Based on their morphological features in comparison with published identification [5,8,34], the collected flukes were found to belong to the superfamily Paramphistomidea. However, in several reports due to unclear morphological boundaries of various genera and species the identification of *Paramphistomum* species required molecular approaches for accurate identification [11,15]. Thus PCR can detect the minute amount of DNA for identifying the genetic fingerprints of *Paramphistome* [18,35]. Several valuable genetic markers including mtDNA and 18S rRNA, have been used effectively to identify trematode species [3]. The 18S rRNA sequences are mostly utilized for *Paramphistomum* species identification [17,36]. Therefore, the 18S rRNA region of collected isolates was amplified through PCR. The collected sample was confirmed *P. cervi* based on 18S rRNA partial fragment with 49% A+T and 51% G+C composition. Previously the full-length sequence of 18S rRNA gene of *P. cervi* was amplified comprises of 47.75% A+T and 52.25% C+G contents [6].

The BLAST analysis of the obtained sequence revealed 100% similarity with *P. cervi* of China and 98% with *P. epiclitum* and other *Paramphistomum* species of India indicating the common phylogenetic history owing to the results of a previous study [25,29]. This similarity may be due to the fact that these areas are neighboring to Pakistan and sharing the same geographical, environmental conditions with free movement of infected hosts. The results are contrary to the report where *P. cervi* was closely related to *Paramphistoma leydeni* [11]. The

genetic evidence for the existence of some other trematodes including *P. epiclitum*, *Gigantocotyle explanatum*, *Fasciola hepatica*, and *Fasciola gigantica* in Pakistan have been previously reported using 18S rRNA markers [1,20,33]. However, for the first time the amplification of 18S rRNA in this study revealed a molecular based evidence for *P. cervi* in Pakistan.

In addition to the molecular approaches, the bioinformatics analysis also provides effective insight into the biology and phylogenetic relations of the parasites [10,31]. The phylogenetic relationship of the generated sequence was analyzed with the similar sequences of trematodes species using the unweighted pair group method with arithmetic mean and evolutionary distances were analyzed using the ML method. Two separate clades and a distinct *T. solium* as outgroup was formed. The phylogenetic analysis of present study isolate forms a monophyletic clade with *P. epiclitum* and other *Paramphistomum* species from India and *P. cervi* from China in a subclade. A separate subclade was made with other trematode species reported from India, China, Saudi Arabia, and Thailand. These similarities indicate that they share the same evolutionary history [2]. A maximum genetic difference was observed with Paramphistomum species of Saudi Arabia [6]. The 18S rRNA sequences of *P. cervi* were found highly conserved [36].

CONCLUSION

For the first time genetic evidence for the presence of *P. cervi* was found in Pakistan showing 100% similarity with the *P. cervi* of China. The data provided 18S rRNA sequence as molecular marker for identification and to study genetic variation among *Paramphistomum* species. The new sequence data will be useful in comparing with other isolates of *Paramphistomum* species from other regions of Pakistan to recover their phylogenetic relationships.

MANUFACTURERS

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***Ethical approval*.** The present study was approved by the advanced studies and research board, Abdul Wali Khan University Mardan.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

REFERENCES

1 **Ali Q., Rashid I., Shabbir M.Z., Akbar H., Shahzad K., Ashraf K. & Chaudhry U. 2018.** First genetic evidence for the presence of the rumen fluke *Paramphistomum epiclitum* in Pakistan. *Parasitology International*. 67(5): 533-537.

2 **Blair D., Campos A., Cummings M.P. & Laclette J.P. 1996.** Evolutionary biology of parasitic platyhelminths: the role of molecular phylogenetics. *Parasitology Today*. 12(2): 66-71.

3 **Chan A.H.E., Chaisiri K., Morand S., Saralamba N. & Thaenkham U. 2020.** Evaluation and utility of mitochondrial ribosomal genes for molecular systematics of parasitic nematodes. *Parasites Vectors*. 13:1-13.

4 **Chandrasekharan K., Radhakrishnan K. & Jacob V.C. 1982.** Efficacy of distodin in the treatment of amphistomiasis in Indian elephants. *Kerala Journal of Veterinary Sciences*. 13(1): 55-58.

5 **Eduardo S.L. 1937.** The taxonomy of the family Paramphistomidae with special reference to the morphology of species occurring in ruminants. *Systematic Parasitology*. 5:25-79.

6 **El-Bahy N.M., Bazh E.K. & Elkhtam A. 2017.** New approach to molecular characterization of *Paramphistomum cervi* and *Carmyerius gregarius* and comparative analyses with selected trematodes. *Parasitology research*. 116(5): 1417-1422.

7 **Firdausy L.W., Prahardani R., Wusahaningtyas L., Soedarmanto I., Wahyu M., Nursalim M.T. & Nurcahyo W. 2019.** Morphological and molecular identification of *Pfenderius heterocaeca* (Trematode: Paramphistomoidea) from Sumatran elephant (*Elephas maximus sumatranus*). *Veterinary World*. 12(8): 1341-1345.

8 **Foreyt W.J. 2001.** Parasites of Cattle, Sheeps and Goats. In: *Veterinary Parasitology* (Reference Manual). 5th edn. Ames: Iowa State Press, pp.69-113.

9 **Godara R., Katoch R., Yadav A. & Rastogi A. 2014.** Epidemiology of paramphistomosis in sheep and goats in Jammu, India. *Journal of Parasitic Diseases*. 38(4): 423-428.

10 **Hernández-Domínguez E.M., Castillo-Ortega L.S., García-Esquivel Y., Mandujano-González V., Díaz-Godínez G. & Álvarez-Cervantes J. 2019.** Bioinformatics as a Tool for the Structural and Evolutionary Analysis of Proteins. *In Phylogenetics*. IntechOpen. DOI: 10.5772/intechopen.89594.

11 **Huson K.M., Oliver N.A. & Robinson M.W. 2017.** Paramphistomosis of ruminants: an emerging parasitic disease in Europe. *Trends in parasitology*. 33(11): 836-844.

12 **Ijaz M., Khan M.S., Avais M., Ashraf K. & Ali M.M. 2008.** Infection rate and chemotherapy of various helminths in goats in and around Lahore. *Pakistan Veterinary Journal*. 28(4): 167-170.

13 **Javed K.U., Akhtar T., Maghbool A. & Anees A. 2006.** Epidemiology of Paramphistomiasis in buffaloes different monumental conditions at four districts in Punjab, Pakistan. Iranian Journal of Veterinary Research. 7(3): 68-72.

14 **Kattak A. 2017.** Prevalence of gastrointestinal parasite, *Paramphistomum* in domestic animals (Cows and Buffaloes) of district Swat and Charsadda, KP, Pakistan. *Journal of Entomology*. 5(3): 907-911.

15 **Khan I., Afshan K., Shah S., Akhtar S., Komal M. & Firasat S. 2019.** Morphological and Molecular Identification of *Paramphistomum epiclitum* from Buffaloes in Pakistan. *Acta Parasitologica*. 65(3): 225-236.

16 **Khedri J., Radfar M.H., Borji H. & Mirzaei M. 2015.** Prevalence and intensity of *Paramphistomum* spp. in cattle from South-Eastern Iran. *Iranian Journal of Parasitology*. 10(2): 268-272.

17 **Lotfy W.M., Brant S.V., Ashmawy K.I., Devkota R., Mkoji G.M. & Loker E.S. 2010.** A molecular approach for identification of paramphistomes from Africa and Asia. *Veterinary Parasitology*. 174(3-4): 234-240.

18 **Ma J., He J.J., Liu G.H., Zhou D.H., Liu J.Z., Liu Y. & Zhu X.Q. 2015.** Mitochondrial and nuclear ribosomal DNA dataset supports that *Paramphistomum leydeni* (Trematoda: Digenea) is a distinct rumen fluke species. *Parasites & vectors*. 8(1): 1-9.

19 **Martinez-Ibeas A.M., Munita M.P., Lawlor K., Sekiya M., Mulcahy G. & Sayers R. 2016.** Rumen fluke in Irish sheep: prevalence, risk factors and molecular identification of two *Paramphistome* species. *BMC Veterinary Research*. 12(1): 1-11.

20 **Mufti S., Afshan K., Khan I.A., Zafar Y., Raza Rizvi S.S., Nazir F. & Qayyum M. 2014.** Genetic Characterization of Fasciola Samples from Bovine Hosts in Pakistan by Sequences of Ribosomal Internal Transcribed Spacer Regions. *Pakistan Veterinary Journal*. 34(3): 361-366.

21 **Patwardhan A., Ray S. & Roy A. 2014.** Molecular markers in phylogenetic studies-a review. *Journal of Phylogenetics & Evolutionary Biology*. 2(2): 1-9.

22 **Prasad P.K., Tandon V., Biswal D.K., Goswami L.M. & Chatterjee A. 2009.** Use of sequence motifs as barcodes and secondary structures of internal transcribed spacer 2 (ITS2, rDNA) for identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae). *Bioinformation*. 3(7): 314-320.

23 **Raza M.A., Murtaza S., Bachaya H.A. & Hussain A. 2009.** Prevalence of *Paramphistomum cervi* in ruminants slaughtered in district Muzaffar Garh. *Pakistan Veterinary Journal*. 28(1): 34-36.

24 **Rehman A., Jingdong L., Chandio A.A. & Hussain I. 2017.** Livestock production and population census in Pakistan: Determining their relationship with agricultural GDP using econometric analysis. *Information Processing in Agriculture*. 4(2): 168-177.

25 **Rinaldi L., Perugini A.G., Capuano F., Fenizia D., Musella V., Veneziano V. & Cringoli G. 2005.** Characterization of the second internal transcribed spacer of ribosomal DNA of *Calicophoron daubneyi* from various hosts and locations in southern Italy. *Veterinary Parasitology*. 131(3-4): 247-253.

26 **Sambrook J. & Russell D.W. 2001.** *Molecular Cloning: a Laboratory Manual*. 3rd edn. New York: Cold Spring Harbor Laboratory Press, pp.23-44.

27 **Samn A.A.A.M. 2017.** A Molecular and Microscopically Studies of *Calicophoron Microbothrium*. *The Egyptian Journal of Hospital Medicine*. 66(1): 28-39.

28 **Shameem H., Devada K. & Lakshmanan B. 2018.** Morphological and molecular characterisation of common amphistome species from cattle of South India. *Journal of Veterinary Medical Allied Sciences*. 2(1): 7-11.

29 **SindiđčM., MartinkovičF., StriškovičT., Špehar M., Štimac I., BujaničM. & KonjevičD. 2017.** Molecular identification of the rumen flukes *Paramphistomum leydeni* and *Paramphistomum cervi* in a concurrent infection of the red deer *Cervus elaphus*. *Journal of Helminthology*. 91(5): 637-641.

30 **Stothard J.R., Hughes S. & Rollinson D. 1996.** Variation within the ribosomal internal transcribed spacer (ITS) from freshwater snails of the genus *Bulinus*. *Acta Tropica*. 61(1): 19-29.

31 Tandon V., Prasad P.K., Chatterjee A. & Bhutia P.T. 2007. Surface fine topography and PCR-based determination of metacercaria of *Paragonimus* sp. from edible crabs in Arunachal Pradesh, Northeast India. *Parasitology Research*. 102(1): 21-28.

32 Titi A., Mekroud A., Chibat H.M., Boucheikhchoukh M., Zein-Eddine R., Djuikwo-Teukeng F.F. & Dreyfuss G. 2014. Ruminal paramphistomosis in cattle from northeastern Algeria: prevalence, parasite burdens and species identification. *Parasite*. 21: 1-8.

33 Ullah S., Afshan K., Arshad M. & Firasat S. 2019. Genetic Characterization of *Gigantocotyle explanatum* from Buffaloes in Northwestern Pakistan. *Kafkas Universitesi Veteriner Fakultesi Dergisi*. 26(2): 225-230. DOI: 10.9775/kvfd.2019.22777.

34 Yamaguti S. 1952. Parasitic worms mainly from Celebes. Part 5. Trematodes of mammals. *Acta Medica Okayama*. 8(4): 341-352.

35 Yamamoto Y. 2002. PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids. *Clinical and Vaccine Immunology*. 9(3): 508-514.

36 Zheng X., Chang Q.C., Zhang Y., Tian S.Q., Lou Y., Duan H. & Zhu X.Q. 2014. Characterization of the complete nuclear ribosomal DNA sequences of *Paramphistomum cervi*. *The Scientific World Journal*. 2014: 1-12.