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Molecular identification of *Fasciola* species based on ribosomal and mitochondrial DNA sequences in Riyadh, Saudi Arabia

[*Identificação molecular de espécies de Fasciola com base emsequências de DNA ribossômico e sequências de DNA ribossômico e mitocondrialemRiad, ArábiaSaudita*]

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ABSTRACT

Fasciola species are the causative agents of liver fluke disease (fasciolosis) which is primarily found in domestic and wild ruminants but also are causal agents of fascioliasis in humans. Few reports for the genotyping of *Fasciola* species in Saudi Arabia were available. This study aimed to identify the *Fasciola* species infecting sheep using morphology and molecular tools. For that, livers of 500 imported sheep were slaughtered at abattoirs located in Riyadh (Saudi Arabia), and parasite species were isolated from 28 liver samples and then preserved for subsequent stages in this study. These parasite species were identified as *Fasciola hepatica* and *Fasciolagigantica* via morphology and confirmed via the 28S rRNA, ITS-1, COI, and NDI sequences with a close identity for other *Fasciola* species within the family Fasciolidae. Phylogenetic analysis of the obtained sequences showed that *Fasciola* isolates from the current study were clustered in one subclade closely related to isolates from Iran, Vietnam, Australia, South Africa, Eastern Europe, India, Egypt, Sudan, Japan, Poland, Spain, Armenia, and Turkey. A combination of data from morphology and molecular analysis could be considered a useful tool for identifying *Fasciola* species infecting sheep in Saudi Arabia.

Keywords: sheep,*Fasciola* species,morphology, molecular analysis

RESUMO

As espécies de Fasciola*sãoosagentescausadores da doença da fascíolahepática (fasciolose), encontradaprincipalmenteemruminantesdomésticos e selvagens, mas tambémsãoagentescausadores da fasciolíaseemhumanos. Haviapoucosrelatos de genotipagem de espécies de FasciolanaArábiaSaudita. O objetivodesteestudofoiidentificar as espécies de Fasciola que infectamovinosusando ferramentas morfológicas e moleculares. Para isso, fígados de 500 ovelhasimportadasforamabatidosemmatadouroslocalizadosemRiad (ArábiaSaudita), e espécies de parasitasforamisoladas de 28 amostras de fígado e preservadas para as etapassubsequentesdesteestudo. Essasespécies de parasitasforamidentificadascomoFasciola hepatica e Fasciolagigantica por meio da morfologia e confirmadas por meio das sequências 28S rRNA, ITS-1, COI e NDI com umaidentidadepróximaaoutrasespécies de Fasciola dentro da famíliaFasciolidae. A análisefilogenética das sequênciasobtidasmostrou que osisolados de Fasciola do presenteestudoforamagrupadosem um subcladointimamenterelacionado a isolados do Irã, Vietnã, Austrália, África do Sul, Europa Oriental, Índia, Egito, Sudão, Japão, Polônia, Espanha, Armênia e Turquia. Uma combinação de dados de morfologia e análise molecular pode ser consideradauma ferramenta útil para identificarespécies de* Fasciola *que infectamovinosnaArábiaSaudita.*

Palavras-chave: ovinos, espécies de Fasciola, *morfologia, análise molecular*

INTRODUCTION

Fasciolosis is considered the most important helminth infection disease in the world. Trematode parasites (liver flukes) are a food-borne zoonotic disease. The two species commonly recognized as the causative agents of fascioliasis in animals and humans are *Fasciola hepatica* and *Fasciolagigantica*(Dorny*et al*., 2009; Bennett *et al*., 2019). Studies have shown that it has affected around 2.4-17 million people,

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with a further 180 million at risk in more than seventy countries around the world, especially where sheep or cattle are reared, that can be transmitted to humans (Erensoy *et al*., 2009; Keiser and Utzinger, 2009; Dar *et al*., 2012; Rokni, 2014). There are certain differences between the geographical distributions of *F. hepatica* and *F. gigantica*. Only *F. hepatica* is distributed in Europe, the Americas, and Oceania, whereas both species are commonly found in Asia and Africa (Mas-Coma *et al*., 2005; Caravedo and Cabada, 2020; Tang *et al*., 2021). Moreover, *F. hepatica* occurs in temperate areas, *F. gigantica* in tropical zones, and both species overlap in subtropical areas and have a global geographical distribution (Lalor*et al*., 2021). Human and animal fascioliasis is a serious health and veterinary problem, of the two species, are a major cause of economic loss to the agricultural community in developing countries, such as weight loss and a decrease in meat production in livestock animals because of morbidity and mortality (Tınar and Korkmaz, 2003).

In the last decades, the prevalence of animal fascioliasis has been investigated in several parts of Saudi Arabia (Khanjari*et al*., 2014). *Fasciola* infection in sheep in some parts of Saudi Arabia is recorded as a very serious problem, where the consumer shifts from local to imported sheep in Riyadh markets (Degheidy and Al-Malki, 2012).

Morphological criteria such as body length to width and shape are among the traditional and important methods to distinguish between the two *Fasciola* species, but these methods are difficult to trust because of the presence of overlapping features and the presence of intermediate forms (Valero *et al*., 2001; Marcilla *et al*., 2002; Periago *et al*., 2006). In Saudi Arabia, animal fascioliasis has been reported with a prevalence of 19.97% and 52.9% in sheep and cattle, respectively. The liver condemnation of meat and offal results in a loss of 0.2 million dollars annually. Human cases of fascioliasis have also been reported among immigrant workers in Saudi Arabia (Sanad and Al-Megrin, 2005; Degheidy and Al-Malki, 2012). In 2020, reported in Europe fascioliasis caused losses of USD 750 million a year, with the largest impact on the dairy and meat cattle industries (Charlier *et al*., 2020).

Recent studies showed that the molecular methods and markers are often necessary for parasite species confirmation and to recognize the intermediate forms (Amor*et al*., 2011; Yuan *et al*., 2016). The nuclear ribosomal DNA (rDNA) is beneficial for molecular studies because it is highly repetitive and contains variable regions flanked by more conserved regions. The first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) which occur between the 18S, 5.8S, and 28S coding regions, have been used for diagnostic purposes at the level of species (Huang *et al*., 2004; Tamura *et al*., 2011). Analysis of mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) and cytochrome *c* oxidase subunit I (COI) DNA sequence variation has been used as a tool to understand the phylogeny of species as well as the geographical distribution of genetic variation and demographic history of populations (Peng*et al*., 2009).

Although significant DNA sequencing for *F. hepatica* and *F. gigantica* is available from several regions, there are few reports for the genotyping of *Fasciola* species in Saudi Arabia. Therefore, our study was conducted to investigate and identify the fasciolid species by morphometric and molecular methods.

MATERIAL AND METHODS

Liver samples of 500 imported sheep (from Sudan and Somalia) were randomly collected from slaughterhouses in Riyadh (Saudi Arabia), during the period of August to November 2022. Macroscopic examination was done for parasitic infections.

Adult trematodes were collected, washed in physiological saline solution (0.9%), and examined to identify morphometric characteristics using described standard taxonomic keys to the species level according to previous studies (Sahba*et al*., 1972; Toft and Eberhard, 1998). *Fasciola* specimens were fixed in 70% ethanol and stored at -80^{o}C until used for molecular identification. The samples were examined and photographed using a Nikon microscope (SMZ18, DS-Ri2, Japan) using NIS Elements software.

Total DNA was extracted from ethanol-preserved samples, and a small part from each ethanol-preserved *Fasciola* specimen was cut, washed in distilled H_2O before DNA isolation, allowed to dry, and crushed in sterile 1.5 ml micro-centrifuge tubes. A portion of the apical and lateral zone of adult flukes, not including the reproductive organs, was removed, and crushed. DNA from the crushed materials was extracted using a QIAGEN DNeasyBlood and Tissue Kit (Catalogue # 69504) according to the manufacturer's instructions. PCR was performed to amplify the target genes, COI, ND1, ITS1, and 28S, using four pairs of primers as shown in Table (1).

Table 1. Sequences of the primers used in PCR reaction

Target	Primers	Sequence	Reference
COI	ITA8	5'-ACGTTGGATCATAAGCGTGT-'3	Itagaki *et al*. (2005)
	ITA9	5'-CCTCATCCAACATAACCTCT-'3	
NDI	ITA10	5'-AAGGATGTTGCTTTGTCGTGG-'3	
	ITA2	5'-GGAGTACGGTTACATTCACA-'3	
ITS1	ITS1-F	5'-TTGCGCTGATTACGTCCCTG-'3	
	ITS1-R	5'-TTGGCTGCGCTCTTCATCGAC-'3	
28S	28SF	5'-ACGTGATTACCCGCTGAACT-'3	Marcilla *et al*. (2002)
	28SR	5'-CTGAGAAAGTGCACTGACAAG-'3	

The polymerase chain reaction (PCR) was performed in 20µL reaction volume, comprising of 4.0µL of Solis BioDyne 5× FIREPolMaster Mix, 12.5mM $MgCl_2$, and 2 mM dNTPs of each, 0.6 µL of each primer, 1.0µL of DNA template and finally 12.8µL of nuclease-free water. PCRs were carried out in a Bio-Rad thermocycler and the thermal cycling conditions involved were an initial denaturation of 3 min at 95°C, followed by 35 cycles of 30 s at 95°C as the denaturation step, an annealing step for 45s at 56°C (28S), 57°C (ITS-1, COI), 60°C (NDI), and an extension for 45s at 72°C. This was followed by a final extension of 5 min at 72°C. Agarose gel electrophoresis was performed to confirm amplification and 1.5% agarose gel was used to separate the PCR products.

All nucleotide sequences were determined through direct sequencing of the PCR products with the ABI Prism BigDye Terminator Cycle Sequencing Kit, using ABI 3130×1 DNA Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA). The sequences were blasted with the GenBank™nucleotide databases. The aligned sequences were trimmed in BIOEDIT 4.8.9 (Hall, 1999). Phylogenetic analysis was performed using the Maximum likelihood, and Tamura-Nei model methods. The software for Molecular Evolutionary Genetics Analysis (MEGA v.11) was used to perform this phylogenetic analysis (Tamura*et al.*, 2021). Boostrap values were included based on 1.000 replicates.

RESULTS

A total of 500 slaughtered sheep were examined, 28 sheep were found to be infected with *F. hepatica*representing 5.6% and *F. gigantica* represented the same percentage.

Microscopic examination showed that hepatic samples were infected with *Fasciola* species, according to the measurements and characteristics of the different *Fasciola* spp., the two *Fasciola* species with different lengths as *F. hepatica* (2.5cm) and *F. gigantica* (5 cm). Both species are characterized by the main diagnostic features such as a flattened body with brown color and leaf-like shape, as well as the presence of two suckers (oral and ventral) (Figure 1). The higher frequency of fascioliasis in imported sheep from Sudan and Somalia is likely because rainfall is higher and irrigation schemes are more developed in that area so that habitats for the intermediate snail hosts are more plentiful there.

PCR products revealed the presence of different bands specified the presence of *Fasciola*species by using the recommended primers as 618 bp (28S rRNA), 680 bp (ITS-1), 500 bp (COI), and 700 bp (NDI) (Figure 2). For analysis of the phylogenetic diversity of the flukes, phylogenetic trees were built, using the partial 28S rRNA, ITS1, COI, and NDI sequences of *F. gigantica* and *F. hepatica* from the present study along with other available sequences from the region,

Asia, Africa and from another region, Europa (Figures 3-6).

Molecular analysis of the partial 28S rRNA region for the recovered specimens revealed the obtaining of 17 sequences which were submitted to GenBank to get accession numbers (OP800250 to OP800266). All sequences are compared with other databases on GenBank and identified as *F. hepatica* and *F. gigantica* within the family Fasciolidae. Phylogenetic analysis revealed that the closest related specimens in the same clade *Fasciola* taxa were collected previously from Iran, Vietnam, Australia, South Africa, and Eastern Europe (Figure 3).

The partial ITS-1 region for the retrieved specimens underwent molecular analysis, and the results showed that 17 specimens were obtained and submitted to the GenBank database to receive accession numbers (OP802570 to OP802574) and (OP866740 to OP866750). All sequences were identified as belonging to the *F. hepatica* and *F. gigantica* species within Fasciolidae after being compared to other databases on GenBank. The most closely related *Fasciola* taxa in the same clade, according to the constructed dendrogram in Figure (4), were previously collected specimens from Iran, Vietnam, India, Egypt, and Japan.

Figure 1. Adult *Fasciola* species recovered in this study, *Fasciola hepatica* (a) and *Fasciolagigantica*(b).

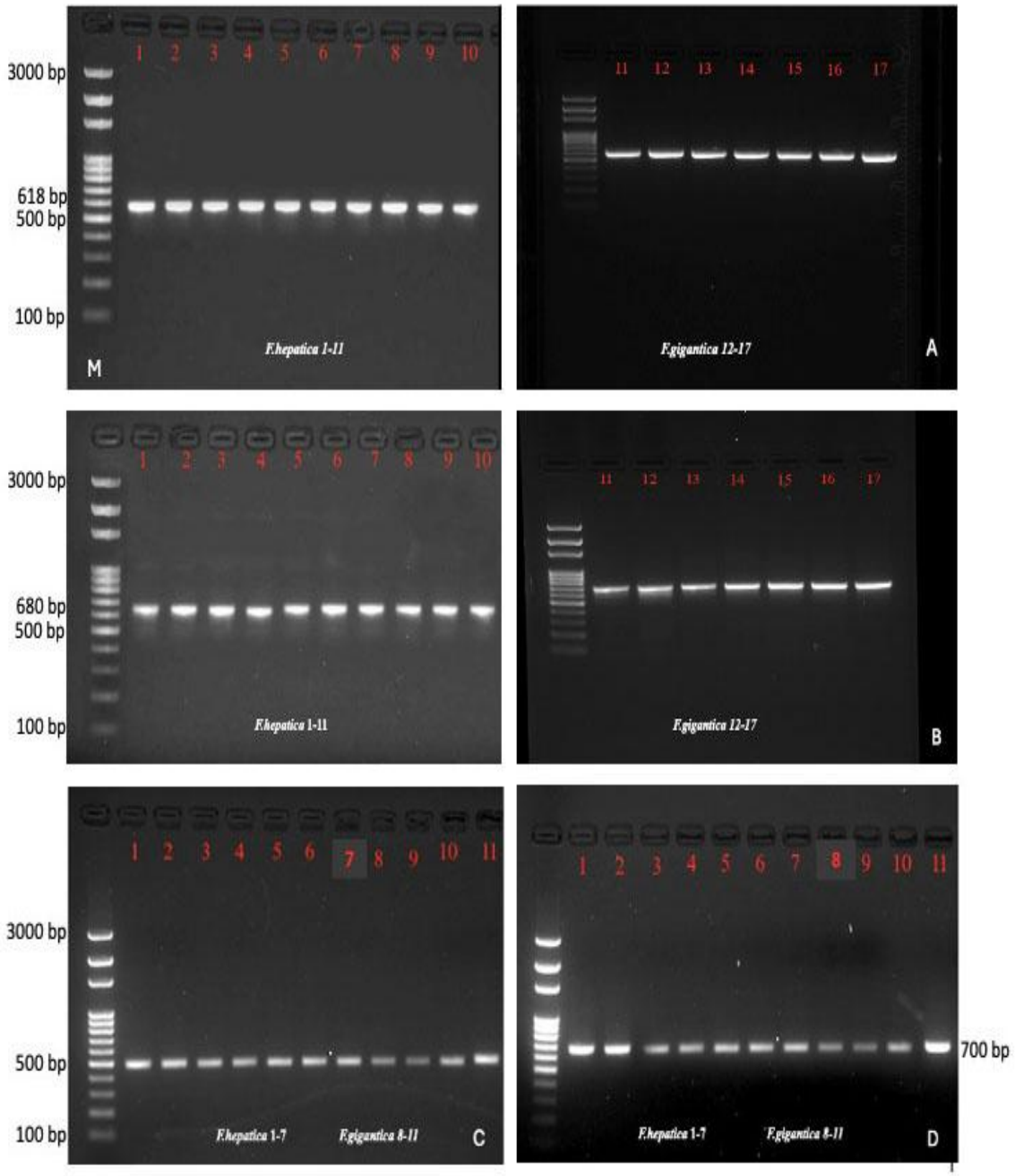

Figure 2. PCR products of 28S rRNA (A), ITS1 (B), COI (C), and NDI (D) genes of *Fasciola* flukes. M: DNA marker (100 bp), Lanes: 1–17 represent different samples of *Fasciola* species.

A molecular investigation of the retrieved specimens with partial COI region revealed that 11 sequences were obtained and submitted to GenBank to receive accession numbers (OP866740 to OP866750). All sequences were identified as belonging to *F. hepatica* and *F. gigantica*species in Fasciolidae after being compared to other databases on GenBank. The most closely related specimens in the same clade of *Fasciola* taxa were previously obtained from Iran, Poland, Spain, Sudan, Egypt, and Japan, according to the phylogeny shown in Figure (5).

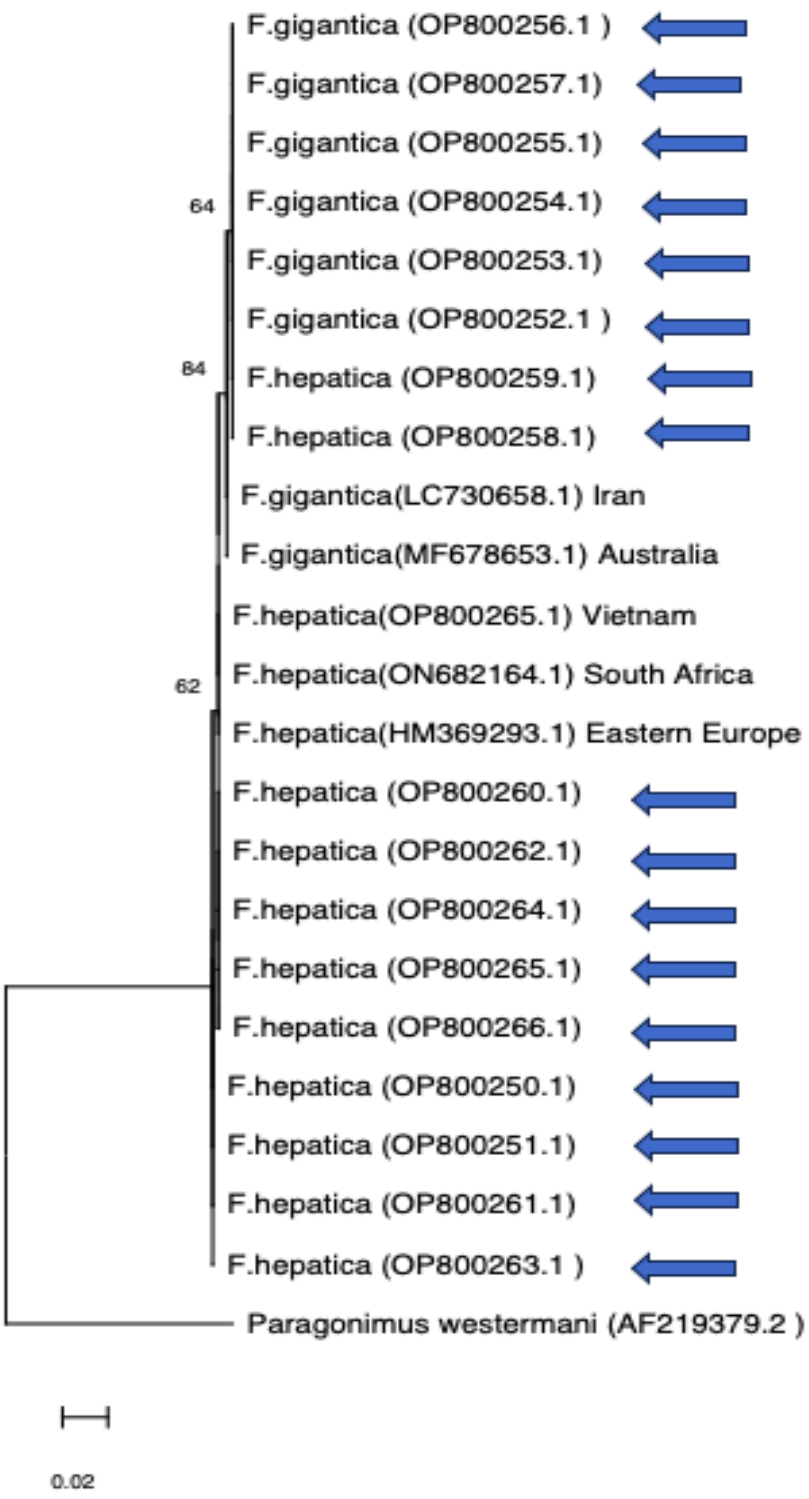

Figure 3. Phylogenetic relationship of 28S sequences of isolates of *F. hepatica* and *F. gigantica* from Saudi Arabia using maximum likelihood method. *P. westermani* as an out-group strain. Numbers at the nodes indicate the percentage of bootstrap support.

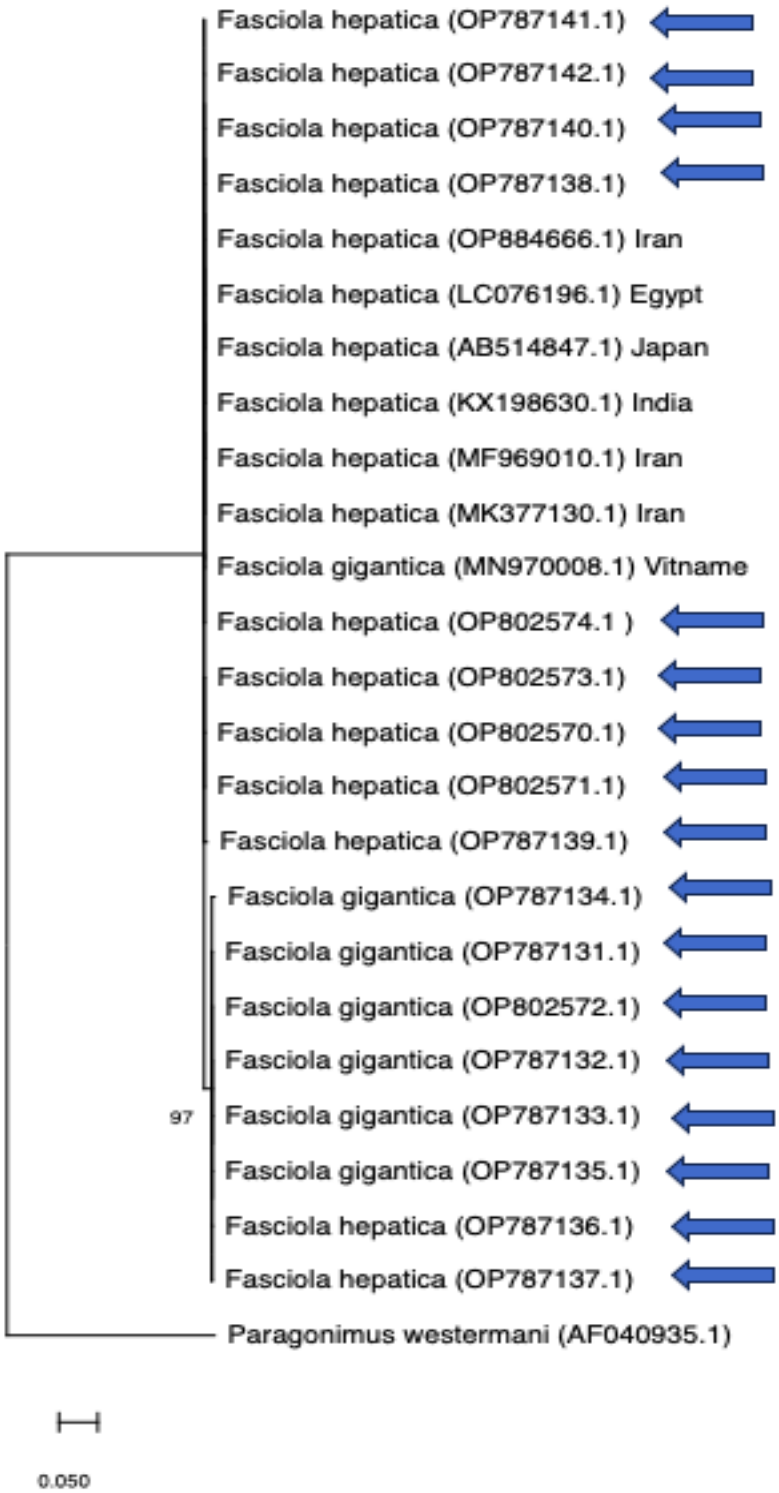

Figure 4. Phylogenetic relationship of ITS1 sequences of isolates of *F. hepatica* and *F. gigantica* from Saudi Arabia using maximum likelihood method. *P. westermani* as an out-group strain. Numbers at the nodes indicate the percentage of bootstrap support.

Molecular analysis of the partial NDI region for the recovered specimens revealed the obtaining of 11 sequences which were submitted to GenBank to get accession numbers (OQ290930 to OQ290940). All sequences are compared with other databases on GenBank and identified as *F. hepatica* and *F. gigantica* within Fasciolidae. Phylogenetic analysis revealed that the closest related specimens in the same clade *Fasciola* taxa were collected previously from Iran, Armenia, Egypt, and Turkey as shown in Figure (6).

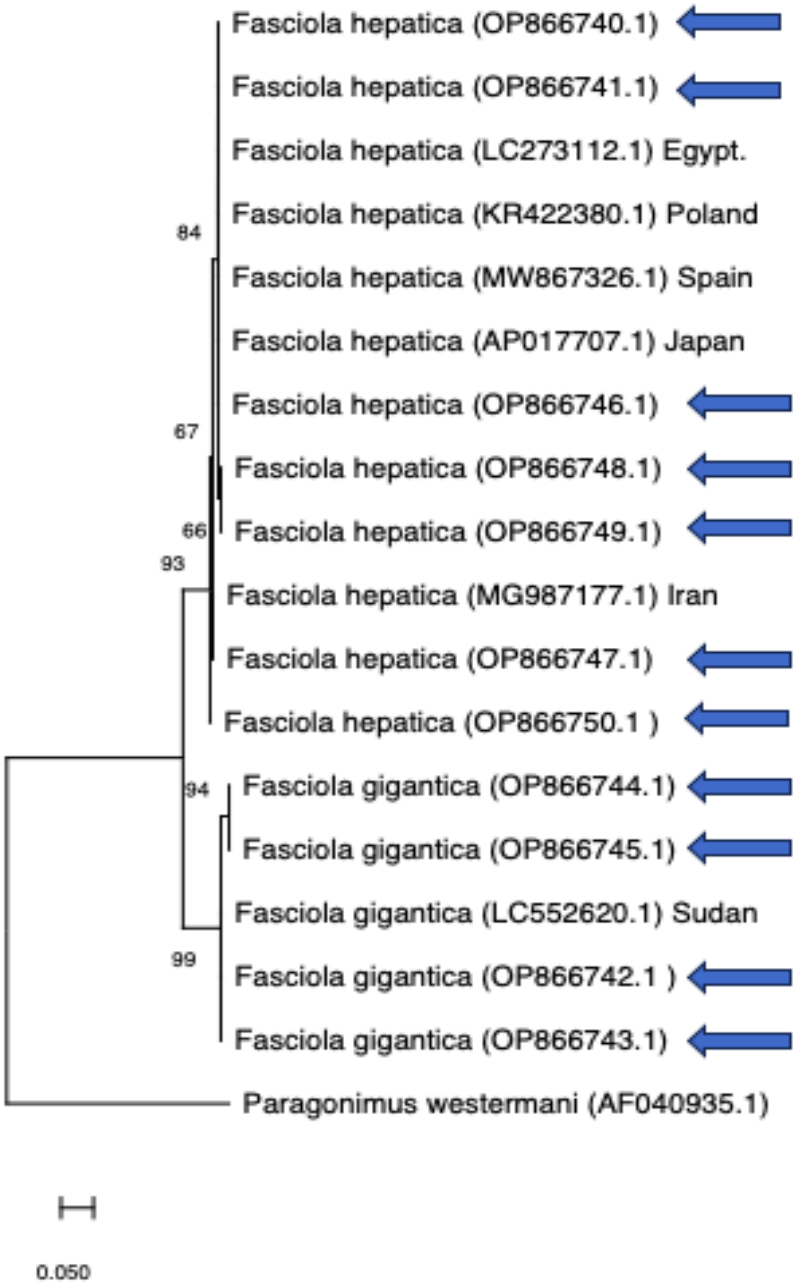

Figure 5. Phylogenetic relationship of COI sequences of isolates of *F. hepatica* and *F. gigantica* from Saudi Arabia using maximum likelihood method. *P. westermani* as an out-group strain. Numbers at the nodes indicate the percentage of bootstrap support.

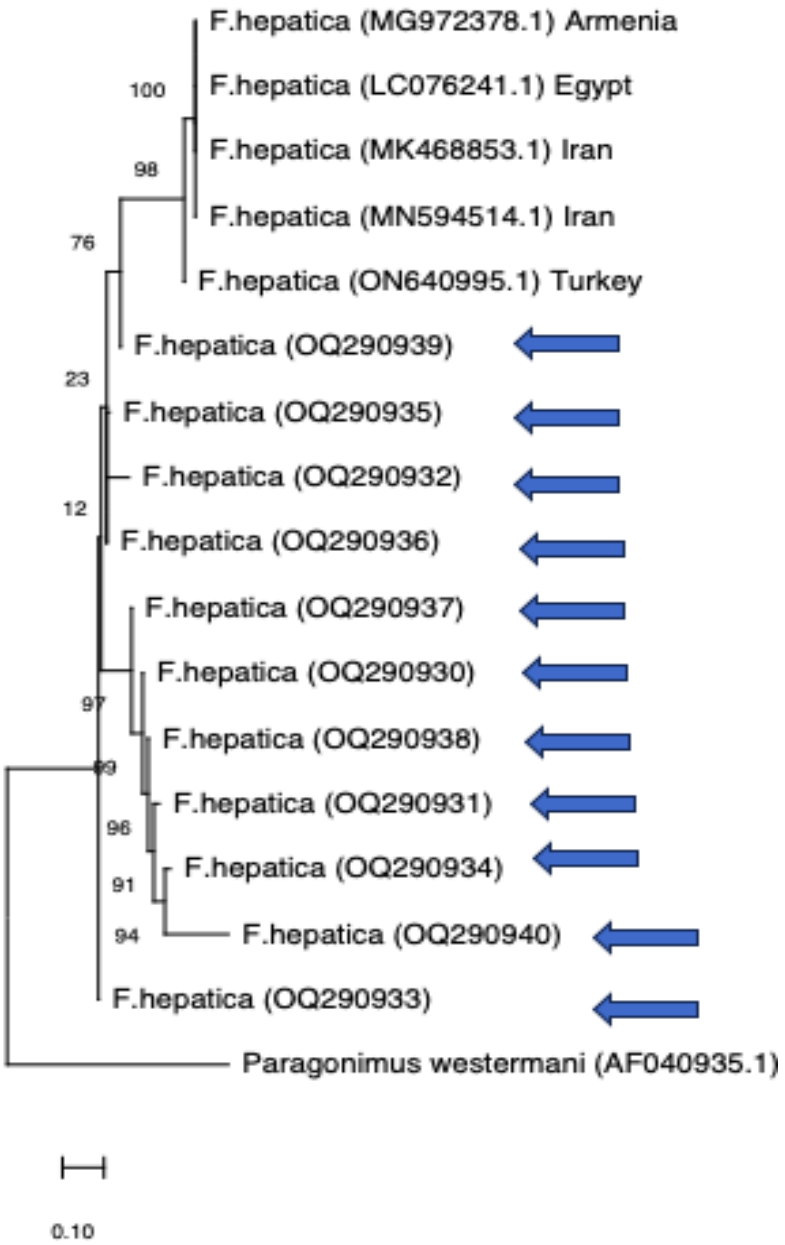

Figure 6. Phylogenetic relationship of NDI sequences of isolates of *F. hepatica* from Saudi Arabia using maximum likelihood method. *P. westermani* as an out-group strain. Numbers at the nodes indicate the percentage of bootstrap support.

DISCUSSION

Fascioliasis control is intricate due to the parasite's complicated life cycle and emerging drug resistance (Kaplan, 2001). To develop effective control measures, it would be necessary to understand the parasite life cycle and evaluate its genetic diversity and population structure (Kaplan, 2001; Itagaki *et al*., 2005). In this study, the natural occurrence of fascioliasis in sheep was reported, and its taxonomic identification via molecular analysis. A total of 28 (5.6%) out of 500 imported sheep were found to be infected with *F. hepatica* and *F. gigantica*. This finding is consistent with Shalaby*et al*. (2013) reporting equal infection rates for both *F.*

hepatica and *F. gigantica* in imported sheep in the Al Taif region in Saudi Arabia. Other previous reports from various Saudi Arabian locations revealed a wide variety of infection rates. In Riyadh, Sanad and Al-Megrin (2005) reported a higher infection rate (21.9%) whereas Degheidy and Al-Malki (2012) reported a 16.3% fasciolosis infection rate. This is inconsistent with Alsulami*et al*. (2022) identified *F. hepatica* as the dominant *Fasciola* species parasitizing isolate from cattle in Jeddah City, Saudi Arabia. In addition, another study showed that infection rates were considerably higher in imported sheep (15.1%) than in domestic sheep (4.96%) (Marcilla *et al*., 2002). Fascioliasis was found in Turkish sheep at rates of 17.55%, 7.90% in Sudanese sheep, 2.3% in Somali sheep, 2.11% in Rumanian sheep, and 0.28% in Australian sheep, according to Shalaby*et al*. (2013). On the other hand, *F. hepatica* was identified by Alajmi (2019) as the main species isolated from sheep in Riyadh, Saudi Arabia.

In comparison with other diagnostic morphological tools for *Fasciola* parasites, molecular methods are more accurate (Olaogun*et al*., 2022). Many studies based on both nuclear and mitochondrial sequences have shown that *F. hepatica* is the species that is most prevalent and abundant in temperate areas, while *F. gigantica* is found in tropical countries of Africa (Alajmi, 2019). In this study, four genes of 28S, ITS-1, COI, and NDI were selected to amplify and identify the recovered parasite species. We employed the genes that evaluate the existing primers targeting eukaryotic. Briefly, the nuclear large subunit rRNA (28S rRNA) has been used for identification, genotyping, intra- and interspecific variations, and phylogenetic studies of these parasites (Conceição *et al*., 2004; Huang *et al*., 2004; Kleiman *et al*., 2007; Prasad *et al*., 2008; Chamuah *et al*., 2016). The first internal transcribed spacers (ITS-1) of nuclear ribosomal DNA (rDNA), have been used for diagnostic purposes at the level of species (Tkach *et al*., 2000; Kostadinova *et al*., 2003; Tandon *et al*., 2007; Ali *et al*., 2008). Mitochondrial DNA such as COI and ND1 has been used to find out the genetic variation of *Fasciola* species (Itagaki *et al*., 2005; Peng *et al*., 2009; Shoriki *et al*., 2014). Based on intra- and interspecific variations between comparable isolates, the recovered *Fasciola* worms were identified as *F. hepatica* and *F. gigantica*(Alajmi, 2019) reported both variations were studied, where interspecific variations range between 0 and 1%, while, variations between studied species were 1%. Similar intraspecific variations were obtained by Galavani*et al*. (2016) in their genetic characterization of *Fasciola* species (*F. hepatica* and *F. gigantic*) isolates from Iran. While interspecific variation reaches 3% by Marcilla *et al*. (2002) which reported few interspecific nucleotide variations between *F. hepatica* and *F. gigantica*. The molecular origin of the Family of Fasciolidae is represented as paraphyletic (Lotfy *et al*., 2008).

CONCLUSION

It could be concluded that there is a low infection with *Fasciola* species in the studied area within Saudi Arabia, also, molecular methods are more effective in identifying these species. Further studies are recommended to control this infection.

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REFERENCES

ALAJMI, R.A. Molecular characterization of *Fasciola* flukes using mitochondrial 28S rRNA gene in Naimi Saudi sheep. *Saudi J. Biol. Sci.*, v.26, p.112-117, 2019.

ALI, H.; AI, L.; SONG, H. *et al.* Genetic characterisation of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. *Parasitol. Res.*, v.102, p.1021-1024, 2008.

ALSULAMI, M.N.; WAKID, M.H.; AL-MATARY, M. *et al.* Identification and genetic characterization of *Fasciola hepatica* isolated from cattle in Jeddah, Saudi Arabia based on sequence analysis of mitochondrial (COI) gene. *Infect. Drug Resist.*, p.4877-4886, 2022.

AMOR, N.; FARJALLAH, S.; SALEM, M. *et al.* Molecular characterization of *Fasciola gigantica* from Mauritania based on mitochondrial and nuclear ribosomal DNA sequences. *Exp. Parasitol.*, v.129, p.127-136, 2011.

BENNETT, J.E.; DOLIN, R.; BLASER, M.J. *Mandell, douglas, and bennett's principles and practice of infectious diseases*. Rio de Janeiro:Elsevier, 2019. (E-book).

CARAVEDO, M.A.; CABADA, M.M. Human fascioliasis: current epidemiological status and strategies for diagnosis, treatment, and control. *Res. Rep. Trop. Med.*, v.26, p.149-158, 2020.

CHAMUAH, J.; RAINA, O.; LALRINKIMA, H. *et al.* Molecular characterization of veterinary important trematode and cestode species in the mithun Bos frontalis from north-east India. *J. Helminthol.*, v.90, p.577-582, 2016.

CHARLIER, J.; RINALDI, L.; MUSELLA, V. *et al.* Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. *Prev. Vet. Med*., v.182, p.105-103, 2020.

CONCEIÇÃO, M.; DURAO, R.; COSTA, I. *et al.*Herd-level seroprevalence of fasciolosis in cattle in north central Portugal. *Vet. P.arasitol*., v.123, p.93-103, 2004.

DAR, Y.; AMER, S.; MERCIER, A. *et al.* Molecular identification of *Fasciola* spp. (digenea: Fasciolidae) in Egypt. *Parasite*, v.19, p.177, 2012.

DEGHEIDY, N.S.; AL-MALKI, J.S. Epidemiological studies of fasciolosis in human and animals at Taif, Saudi Arabia. *WorldAppl. Sci. J*., v.19, p.1099-1104, 2012.

DORNY, P.; PRAET, N.; DECKERS, N. *et al.* Emerging food-borne parasites. *Vet. Parasitol.*, v.163, p.196-206, 2009.

ERENSOY, A.; KUK, S.; OZDEN, M. Genetic identification of *Fasciola hepatica* by ITS-2 sequence of nuclear ribosomal DNA in Turkey. *Parasitol. Res.*, v.105, p.407-412, 2009.

GALAVANI, H.; GHOLIZADEH, S.; TAPPEH, K.H. Genetic characterization of *Fasciola* isolates from West Azerbaijan province Iran based on ITS1 and ITS2 sequence of ribosomal DNA. *Iran. J. Parasitol.*, v.11, p.52, 2016.

HALL, T.A. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.*, v.41, p.95-98, 1999.

HUANG, W.; HE, B.; WANG, C. *et al.* Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. *Vet. Parasitol.*, v.120, p.75-83, 2004.

ITAGAKI, T.; KIKAWA, M.; TERASAKI, K. Molecular characterization of parthenogenic *Fasciola* sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. *J. Vet. Med. Sci.*, v.67, p.1115-1118, 2005.

KAPLAN, R.M. *Fasciola hepatica*: a review of the economic impact in cattle and considerations for control. *Vet. Ther.*, v.2, p.40-50, 2001.

KEISER, J.; UTZINGER, J.R. Food-borne trematodiases. *Clin. Microbiol. Rev.*, v.22, p.466-483, 2009.

KHANJARI, A.; BAHONAR, A.; FALLAH, S. *et al*. Prevalence of fasciolosis and dicrocoeliosis in slaughtered sheep and goats in Amol Abattoir, Mazandaran, northern Iran. *Asian Pac. J. Trop. Dis.*, v.4, p.120-124, 2014.

KLEIMAN, F.; PIETROKOVSKY, S.; PREPELITCHI, L. *et al.* Dynamics of *Fasciola hepatica* transmission in the Andean Patagonian valleys, Argentina. *Vet. Parasitol.*, v.145, p.274-286, 2007.

KOSTADINOVA, A.; HERNIOU, E.; BARRETT, J. *et al.*Phylogenetic relationships of *Echinostoma* Rudolphi, 1809 (Digenea: Echinostomatidae) and related genera re-assessed via DNA and morphological analyses. *Sys. Parasitol.*, v.54, p.159-176, 2003.

LALOR, R.; CWIKLINSKI, K.; CALVANI, N.E.D. *et al.* Pathogenicity and virulence of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* that cause the zoonosis Fasciolosis. *Virulence*, v.12, p.2839-2867, 2021.

LOTFY, W.M.; BRANT, S.V.; DEJONG, R.J. *et al.* Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae). *Am. J. Trop. Med. Hyg.*, v.79, p.248, 2008.

MARCILLA, A.; BARGUES, M.; MAS-COMA, S. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Mol. Cell. Probes*, v.16, p.327-333, 2002.

MAS-COMA, S.; BARGUES, M. D.; VALERO, M. Fascioliasis and other plant-borne trematode zoonoses. *Inter. J. Parasitol.*, v.35, p.1255-1278, 2005.

OLAOGUN, S.C.; BYARUHANGA, C.; OCHAI, S.O. *et al.*Comparison of three diagnostic methods to detect the occurrence of Fasciola species in communally grazed cattle in the North West Province, South Africa. *Pathogens*, v.11, p.1398, 2022.

PENG, M.; ICHINOMIYA, M.; OHTORI, M. *et al.* Molecular characterization of *Fasciola hepatica*, *Fasciola gigantica*, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. *Parasitol. Res.*, v.105, p.809-815, 2009.

PERIAGO, M.; VALERO, M.; PANOVA, M. *et al.*Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). *Parasitol. Res.*, v.99, p.368-378, 2006.

PRASAD, P.; TANDON, V.; BISWAL, D. *et al.* Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions. *Parasitol. Res.*, v.103, p.1247-1255, 2008.

ROKNI, M. Helminth-Trematode: *Fasciola hepatica* and *Fasciola gigantica*. *Encycl. Food Saf.*, v.2, p.140-145, 2014.

SAHBA, G.H.; ARFAA, F.; FARAHMANDIAN, I. *et al.* Animal fascioliasis in Khuzestan, southwestern Iran. *J. Parasitol*., v.58, p.712-716, 1972.

SANAD, M.M.; AL-MEGRIN, W.A. Fascioliasis among local and imported sheep in Saudi Arabia: parasitological and serological diagnosis. *J. Egypt. Soc. Parasitol*., v.35, p.1121-1134, 2005.

SHALABY, I.; GHERBAWY, Y.; BANAJA, A. Molecular characterization of *Fasciola* species isolated from imported sheep in Taif region (Saudi Arabia). *Trop. Biomed.*, v.30, p.15-26, 2013.

SHORIKI, T.; ICHIKAWA-SEKI, M.; DEVKOTA, B. *et al.* Molecular phylogenetic identification of *Fasciola* flukes in Nepal. *Parasitol. Int.*, v.63, p.758-762, 2014.

TAMURA, K.; PETERSON, D.; PETERSON, N. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*., v.28, p.2731-2739, 2011.

TAMURA, K.; STECHER, G.; KUMAR, S. MEGA11: molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol*., v.38, p.3022-3027, 2021.

TANDON, V.; PRASAD, P.; CHATTERJEE, A. *et al.* Surface fine topography and PCR-based determination of metacercaria of *Paragonimus* sp. from edible crabs in Arunachal Pradesh, Northeast India. *Parasitol. Res.*, v.102, p.21-28, 2007.

TANG, M.; ZHOU, Y.; LIU, Y. *et al.*Molecular identification and genetic-polymorphism analysis of *Fasciola* flukes in Dali Prefecture, Yunnan Province, China. *Parasitol. Int.*, v.85, p.102416, 2021.

TINAR, R.; KORKMAZ, M. *Fasciolosis*. Turkey: Parasitology Association, 2003. Publication 18.

TKACH, V. V., PAWLOWSKI, J., SHARPILO, V. P. Molecular and morphological differentiation between species of the *Plagiorchis vespertilionis* group (Digenea, Plagiorchiidae) occurring in European bats, with a re-description of *P. vespertilionis* (Müller, 1780). *Sys. Parasitol.*, v.47, p.9-22, 2000.

TOFT, J.D.; EBERHARD, M.L. Parasitic diseases. In: YOST, O.C.; AUTUMN, D.; KENNETH, E.; RAMO, S. (Eds.).*Nonhuman primates in biomedical research* Washington: Elsevier, 1998. p.111-205.

VALERO, M.A.; DARCE, N.A.N.; PANOVA, M. *et al.*Relationships between host species and morphometric patterns in Fasciola hepatica adults and eggs from the Northern Bolivian Altiplano hyperendemic region. *Vet. Parasitol.*, v.102, p.85-100, 2001.

YUAN, W.; LIU, J.M.; LU, K. *et al.* Molecular identification and seasonal infections of species of *Fasciola* in ruminants from two provinces in China. *J. Helminthol*., v.90, p.359-363, 2016.