Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 4, 659-662

Molecular identification of the worm *Fasciola* sp. on cattle at the Kediri City Slaughterhouse, Indonesia

Firdha H. Nifa[1], Poedji Hastutiek[2], Kusnoto[2*], Suhita Aryaloka[3], Aswin R. Khairullah[4], Shendy C. Kurniawan[5], Abdullah Hasib[6], Candra D. Atma[7], Siti R. Ayuti[8], Ertika F. Lisnanti[9]

[1]*Master Program of Veterinary Science and Public Health, Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Dr. Ir. H. Soekarno, Kampus C. Mulyorejo, Surabaya 60115, East Java, Indonesia.*
[2]*Division of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Dr. Ir. H. Soekarno, Kampus C Mulyorejo, Surabaya 60115, East Java, Indonesia.*
[3]*Master Program of Veterinary Agribusiness, Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Dr. Ir. H. Soekarno, Kampus C Mulyorejo, Surabaya 60115, East Java, Indonesia.*
[4]*Research Center for Veterinary Science, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 46 Cibinong, Bogor 16911, West Java, Indonesia.*
[5]*Master Program of Animal Sciences, Department of Animal Sciences, Specialization in Molecule, Cell and Organ Functioning, Wageningen University and Research, Wageningen 6708 PB, Netherlands.*
[6]*School of Agriculture and Food Sustainability, The University of Queensland, Gatton, QLD 4343, Queensland, Australia.*
[7]*Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, Universitas Pendidikan Mandalika, Jl. Pemuda No. 59A, Dasan Agung Baru, Mataram 83125, West Nusa Tenggara, Indonesia.*
[8]*Faculty of Veterinary Medicine, Universitas Syiah Kuala, Jl. Teuku Nyak Arief No. 441, Kopelma Darussalam, Banda Aceh 23111, Aceh, Indonesia.*
[9]*Program of Animal Husbandry, Faculty of Agriculture, Universitas Islam Kediri, Jl. Sersan Suharmaji No.38, Manisrenggo, Kediri 64128, East Java, Indonesia.*

ARTICLE INFO

Recieved: 02 January 2024

Accepted: 06 March 2024

***Correspondence:**

Corresponding author: Kusnoto
E-mail address: kusnotokk58@gmail.com

Keywords:

Fasciola sp.
Cattle
PCR
Slaughterhouse
Public health

ABSTRACT

Fasciolosis is a disease caused by the genus *Fasciola* which attacks wild animals, livestock and humans. Molecular characterization of *Fasciola* sp. important for identifying the species *Fasciola* sp. The aim of this research was to analyze the characteristics of the worm *Fasciola* sp. molecularly on samples of cattle slaughtered at the Kediri City Slaughterhouse Regional Technical Implementation Unit. This research was carried out from January to February 2023. The samples were adult worms *Fasciola* sp. taken from one beef cattle and one dairy cattle. The PCR used is conventional PCR with primers from mitochondrial DNA genes. PCR products are passed to the sequencing stage. The PCR test results read on 2% agarose gel electrophoresis showed that the PCR product had high specificity, namely forming a single band at position 752 bp showed positive for *F. gigantica*. the results of the phylogenetic tree of *Fasciola* sp. with data in GenBank showing *Fasciola* sp. beef and dairy cattle isolates in Kediri City are closely related to *Fasciola* sp. dairy cattle isolate from China with Accession Number KF543343.1 and *Fasciola* sp. long-haired cattle (yak) isolate from China Accession Number MH621335.1. The existence of international livestock trade can result in the introduction of various diseases, one of which is Fasciolosis. Livestock import-export activities from China allow the introduction of *Fasciola* sp. to Indonesia.

Introduction

Fasciolosis is a disease caused by the genus *Fasciola* which attacks wild animals, livestock, and humans (Lalor *et al*., 2021). This disease has a major impact on reducing livestock productivity. *Fasciola hepatica* and *Fasciola gigantica* are the species most frequently found in Fasciolosis cases (Rokni *et al*., 2018; Silva *et al*., 2020). This disease is included in the Human Neglected Tropical Diseases (NTDs) category by the World Health Organization (WHO) (Angles *et al*., 2022). Globally, it is estimated that around 17 million people are infected with fasciolosis with severe symptoms (Caravedo and Cabada, 2020). The species *Fasciola* sp. has a very wide geographical distribution from subarctic areas to tropical areas. *Fasciola* sp. has genetic material and phenotype that resembles *F. hepatica* and *F. gigantica*.

The detrimental presence of *Fasciola* sp. is caused by livestock traffic which causes the carrying of *Fasciola* sp. from one country to another (Nasreldin and Zaki, 2020). The transfer of parasites from endemic areas to non-endemic areas causes the distribution of *Fasciola* sp. to become unclear and encourages sympatric speciation (Le *et al*., 2008). Sympatric speciation is the existence of two or more types of a species in the same geographic conditions and time (McCoy, 2003). This sympatric speciation encourages hybridization and forms intermediate organisms from the two species which are usually referred to as *Fasciola* sp. (Saijuntha *et al*., 2018). *Fasciola* sp. morphometrically it can resemble *F. hepatica* and *F. gigantica* and the three species of *Fasciola* sp. this is detrimental.

Carcass weight of cattle not infected by *Fasciola* sp. will be higher than those infected with *Fasciola* sp (Opio *et al*., 2021). This difference in carcass weight is usually found at young ages up to 30 months. The difference in carcass weight at the age of 23-30 months is estimated at 6.34 kg (da Costa *et al*., 2019). In addition, carcasses from infected cattle have less fat content compared to carcasses from healthy cattle (Oehm *et al*., 2023). Therefore, the emergence of Fasciolosis is detrimental to the livestock industry. Research on the molecular identification of *Fasciola* especially *Fasciola* sp. has been carried out in China, Vietnam, and Thailand (Anh *et al*., 2018; Zhang *et al*., 2019a; Thanasuwan and Tankrathok, 2021).

Molecular characterization of *Fasciola* sp. important for identifying the species *Fasciola* sp. Information regarding the phenotype and molecular characters of *Fasciola* sp. very useful for accurately identifying the etiology and epidemiology of fasciolosis in each endemic area (Alsulami *et al*., 2023). This species difference is important to know because the intermediate hosts for each species are different ecologically and biologically. One of the molecular methods developed to differentiate the species *Fasciola* sp. is the Polymerase Chain Reaction (PCR) method. The PCR method is a method used to amplify and reproduce certain nucleotide sequences. The PCR method has higher sensitivity when compared to morphological examination (Bankur *et al*., 2014).

The Kediri City Slaughterhouse Regional Technical Implementation Unit is one of the largest slaughterhouses in Indonesia. Molecular examination related to *Fasciola* sp. This has never been done even though many cattles are slaughtered at the slaughterhouse. This research uses

ISSN: 2090-6277/2090-6269/

one of the genes from the mitochondrial DNA (mtDNA) genome, namely the *nad4* gene. The mtDNA genome is used because almost all eukaryotes have mitochondrial genomes that evolve more rapidly than nuclear genomes (Dowling and Wolff, 2023). Therefore, these mitochondrial genes are very suitable for distinguishing species that are quite closely related or whose differences between species are relatively small at the sub-species level (Bocek *et al*., 2019). The results of mtDNA genome sequencing can also be used to prove the existence of *Fasciola* sp.

Fasciola sp. infection in cattle can be a public health problem. The aim of this research was to analyze the characteristics of the worm *Fasciola* sp. molecularly on samples of cattle slaughtered at the Kediri City Slaughterhouse Regional Technical Implementation Unit. Through this research, it is hoped that we can find out the extent of the distribution of *Fasciola* sp. experienced overlap in cattle raised in Indonesia.

Materials and methods

Sample collection

This research was carried out from January to February 2023. The samples were adult worms *Fasciola* sp. taken from one beef cattle and one dairy cattle slaughtered at the Kediri City Slaughterhouse Regional Technical Implementation Unit, then the samples obtained were collected in a 100 cc plastic pot containing physiological NaCl. Each sample was labeled with the cow's region of origin, cow breed, and sex, then stored in an ice box at 4°C.

DNA extraction of Fasciola sp.

Sample preparation is carried out by taking the worms to be identified and then chopping them finely using a scalpel. Then put the worms into a 1.5 ml tube. The solution was added with 20 µl of Qiagen Protease, 10 µl of lysozyme enzyme and 180 µl of ATL buffer. The sample was then vortexed for 15 seconds and spindown for approximately 3 seconds. Samples were incubated at 60°C for 30 minutes. The sample was then added with 200 µl of AL buffer into the sample, then vortexed for 15 seconds. The sample was then added with 200 µl of 96% ethanol and mixed by vortexing for 15 seconds, then spindown. The mixture was then put into a QIAamp® Mini spin column (2 ml collection tube) and centrifuged at 8,000 rpm for 1 minute.

Remove 2 ml of the filtrate from the collection tube and replace it with a new 2 ml collection tube. The solution was then added with 500 µl of Buffer AW1, then centrifuged at 8000 rpm for 1 minute. 2 ml of the solution filtrate was discarded and replaced with a new 2 ml collection tube and 500 µl of Buffer AW2 was added, then centrifuged at 13,000 rpm for 3 minutes. Then 2 ml of the filtrate was discarded and replaced with a new 2 ml collection tube. Centrifuge again at 13,000 rpm for 1 minute. The QIAamp® Mini spin column was transferred to a 1.5 ml microcentrifuge tube. Then 60 µl of Buffer AE or distilled water was added. Incubate at room temperature (15-25°C) for 1 minute then centrifuge at 8,000 rpm for 1 minute.

Polymerase Chain Reaction technique

The PCR used is conventional PCR with primers from mitochondrial DNA genes. The two pairs of primers used in this study have the arrangement as in Table 1. Polymerase Chain Reaction (PCR) steps are carried out by mixing 25 µl 2x MyTaq™ HS Red Mix, 5 µl DNA template, 1 µl primer F, 1 µl primer R and 18 µl of distilled water with a total volume of 50 µl were put into a microtube then vortexed. The microtubes are then inserted into a thermo cycle machine using a program consisting of predenaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C. for 30 seconds, then repeated for 35 cycles and final extension at 72°C for 5 minutes.

Table 1. Primer arrangement used in the PCR method.

Species	Primer	Base pair	Reference
F. hepatica	F: 5′-GCT TGT TTG GCA TTG TTAGGG-3′ R: 5′-CAA CCA GCC CAT CAA TCCC-3′	304 bp	Ayaz *et al*. (2014)
F. gigantica	F: 5′-GGGAT TCA GTC TTG GAG GGA-3′ R: 5′-CCG CCA TAA ACA CCA CAC CT-3′	752 bp	Le *et al*. (2012)

F: Forward; R: Reverse

Electrophoresis

Analysis of PCR results was carried out by 2% (w/v) agarose gel electrophoresis using an electrophoresis apparatus. Gel molds were made by filling 20 ml of PBS with an agar concentration of 2%. Weigh 0.4 grams of agarose then put it in 20 ml of PBS then heat it in the microwave for±2 minutes (until completely dissolved). The solution was then added with 2 µl of RedSafe™ gel dye and mixed until homogeneous. The next step is to put the solution into the mold according to the size and wait until it solidifies. The next step is electrophoresis. Electrophoresis was carried out in TBE 1X buffer as a current-conducting medium at a voltage of 100 Volts and a current of 250 Amperes for 30 minutes. The results of electrophoresis were then visualized using a Transluv illuminator and documented.

Sequencing

PCR products were purified using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermofisher Scientific) purification kit and then sequenced using forward primers in an ABI PRISM® 3730xl DNA Analyzer (Thermofisher Scientific) automatic DNA sequencer. The nucleotide sequence was read using BioEdit Sequence Alignment Editor software and analyzed for the level of nucleotide similarity (homology) using the NCBI-BLAST (National Center of Biotechnology Information – Basic Local Alignment Search Tool) website on GenBank.

Results

Worm *Fasciola* sp. which infected beef cattle at the Kediri City Slaughterhouse had a body length of 19.9-27.3 mm with an average body length of 23.82±3.12 mm and a body width of 5.2-7.9 mm with an average 6.68±1.24 mm. Meanwhile, the worm *Fasciola* sp. those that infect dairy cattle have a body length of 21.4-32.5 mm with an average of 25±4.61 mm and a body width of 5.2-7.8 mm with an average of 6.4±0.97 mm. The morphological form of the *Fasciola* sp. worm can be seen in Figure 1.

Fig. 1. Morphological form of *Fasciola* sp. in cattle.

The PCR test results read on 2% agarose gel electrophoresis showed that the PCR product had high specificity, namely forming a single band at position 752 bp according to the amplification target which can be seen in Figure 2. Sample number 1 showed positive for *F. gigantica* from beef cattle and while the sample sample number 2 showed positive for *F. gigantica* from dairy cattle. Sample number 3 showed positive for *F. hepatica* from beef cattle and sample number 4 showed positive for *F. hepatica* from dairy cattle.

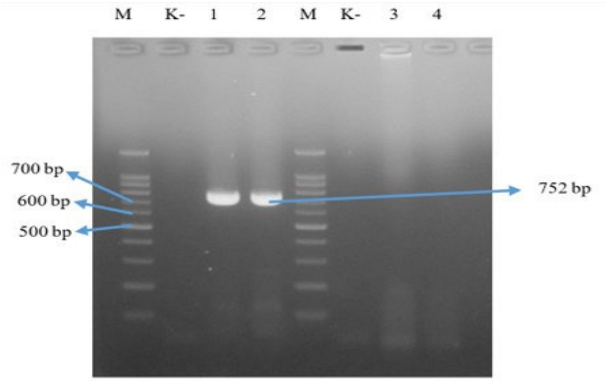

Fig. 2. PCR product electrophoresis results.

Multiple alignments of the nucleotide sequences of the isolates studied showed the presence of different or changed nitrogen bases and fixed or similar sequences between isolates. The mutation that occurred in the sample from the Kediri City Slaughterhouse was a substitution. Substitution mutations that occurred in samples of *Fasciola* sp. from beef cattle are at base positions 57 (A-G), 75 (A-G), 216 (G-A), 279 (A-G), 286 (C-T), 348 (C-T), 350 (T-A), 351 (A-T), 353 (T-C), 358 (C-G), 359 (T-G), 362 (G-A), 370 (A-T), 420 (T-C), 465 (A-G), 573 (A-G), 618 (C-G), 634 (T-A), and 639 (A-G). Substitution mutations that occurred in samples of *Fasciola* sp. from dairy cows are at base positions 57 (A-G), 75 (A-G), 216 (G-A), 279 (A-G), 286 (C-T), 348 (C-T), 350 (T-A), 351 (A-T), 353 (T-C), 358 (C-G), 359 (T-G), 362 (G-A), 370 (A-T), 420 (T-C), 465 (A-G), 573 (A-G), 607 (T-A), 618 (C-G), 623 (T-A), 631 (T-G), 634 (T-A), and 639 (A-G). Nitrogen base position of the *nad4* coding gene *Fasciola* sp. can be seen in Table 2.

Table 2. Nitrogen base position of the *nad4* coding gene *Fasciola* sp. a substitution mutation occurs.

Mutation	*Fasciola* sp. from dairy cattle	*Fasciola* sp. from dairy cow
Substitution	57 (A-G)	57 (A-G)
	75 (A-G)	75 (A-G)
	216 (G-A)	216 (G-A)
	279 (A-G)	279 (A-G)
	286 (C-T)	286 (C-T)
	348 (C-T)	348 (C-T)
	350 (T-A)	350 (T-A)
	351 (A-T)	351 (A-T)
	353 (T-C)	353 (T-C)
	358 (C-G)	358 (C-G)
	359 (T-G)	359 (T-G)
	362 (G-A)	362 (G-A)
	370 (A-T)	370 (A-T)
	420 (T-C)	420 (T-C)
	465 (A-G)	465 (A-G)
	573 (A-G)	573 (A-G)
		607 (T-A)
	618 (C-G)	618 (C-G)
		623 (T-A)
		631 (T-G)
	634 (T-A)	634 (T-A)
	639 (A-G)	639 (A-G)

In Figure 3, the results of the phylogenetic tree of *Fasciola* sp. with data in GenBank showing *Fasciola* sp. beef and dairy cattle isolates in Kediri City are closely related to *Fasciola* sp. dairy cattle isolate from China with Accession Number KF543343.1 and *Fasciola* sp. long-haired cattle (yak) isolate from China Accession Number MH621335.1. The results of homology analysis of samples from beef cattle and dairy cattle compared with reference isolates from GenBank showed closeness with the lowest percentage of 95% and the highest percentage of 99% for each sample and sequence data obtained from GenBank as can be seen in Table 3.

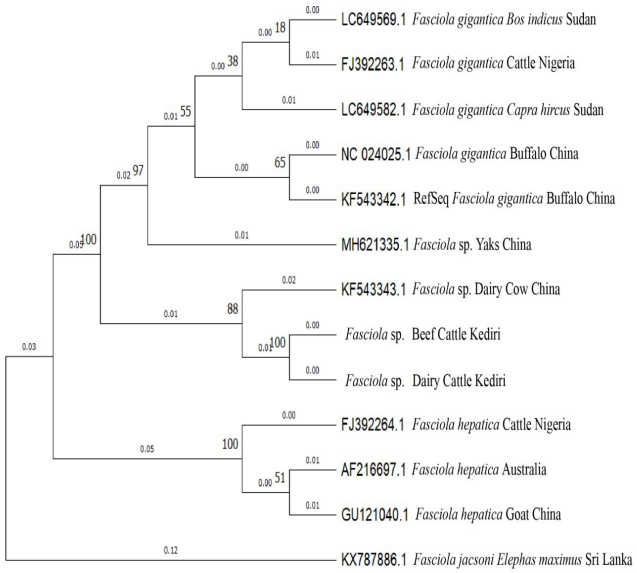

Fig. 3. Phylogenetic tree of *Fasciola* sp. Isolates.

Table 3. Homology analysis of the *nad4* gene encoding *Fasciola* sp.

Homology	1	2	3	4	5	6	7	8	9
Beef cattle (1)	100								
Dairy cattle (2)	99	100							
KF543343.1 (3)	97	97	100						
MH621335.1 (4)	96	95	98	100					
LC649569.1 (5)	96	96	97	98	100				
GU121036.1 (6)	98	98	99	98	96	100			
FJ392263.1 (7)	97	97	95	96	99	96	100		
LC649582.1 (8)	96	95	97	97	99	95	98	100	
NC024025.1 (9)	96	95	97	98	97	98	97	97	100

Discussion

Fasciolosis is an important zoonotic parasitic disease that can infect animals and humans worldwide. This disease is caused by ingestion of infective metacercariae from the worms *F. gigantica* and *F. hepatica* (Lalor *et al*., 2021). *Fasciola* sp. infection in both humans and animals through the same mechanism, namely through contaminated food/water (Cwiklinski *et al*., 2016). Currently, fasciolosis infection has been reported in 81 countries around the world (Birlutiu and Birlutiu, 2023). Around 2.4 to 17 million people in the world are infected with fasciolosis (Caravedo and Cabada, 2020).

Fasciola sp. has a complex cycle involving intermediate hosts, namely water snails, and definitive hosts, namely mammals, including humans (Zhang *et al*., 2019b). The eggs are excreted with the feces of the definitive and adult hosts in the environment and form a miracidium. The miracidium goes out looking for an intermediate host (Beesley *et al*., 2018). Snails that are suitable for miracidium development are snails from the Lymnaeidae family. Distribution of the species *Fasciola* sp. in the world is influenced by the distribution of intermediate hosts where infective larvae of *Fasciola* sp (Nyagura *et al*., 2022).

Distribution of *Fasciola* sp. influenced by climate and rainfall changes in some areas and can develop in new areas that are suitable for its development (Fox *et al*., 2011). Human activities such as migration, globalization, exports and imports of live animals have the potential to bring *Fasciola* sp. from one place to another (Calvani and Šlapeta, 2021). This activity resulted in the distribution of *Fasciola* sp. becomes unclear and extends from endemic areas.

PCR method on adult worms *Fasciola* sp. often use ribosomal DNA

genes such as ITS1 and ITS2, as well as mitochondrial genes such as nad and cox (Saadatnia *et al*., 2022). ITS1 and ITS2 regions in *Fasciola* sp. located in the large and small nuclear rRNA subunits which can provide genetic markers for the identification of *Fasciola* sp. at the species level (Tadayon *et al*., 2015). In the ITS2 sequence, as many as six nucleotide sites are different in the *F. hepatica* and *F. gigantica* species, one of which is due to a deletion in this area in the *F. gigantica* species (Raina *et al*., 2015). *Fasciola* sp. carries nucleotides in the ITS1 and ITS2 regions originating from both species.

In this study, mitochondrial genome DNA (mtDNA) was used to identify the two *Fasciola* species, namely *F. gigantica* and *F. hepatica*. The mtDNA genome is used because almost all eukaryotes have a mitochondrial genome which evolves faster than nuclear genes and therefore, the mitochondrial genome is very suitable for distinguishing species that are quite closely related or where the differences between species are relatively small down to the sub-species level (Gao *et al*., 2022). The results of mtDNA genome sequencing can also be used to prove the existence of *Fasciola* sp.

The results of the phylogenetic tree show that *Fasciola* sp. isolates from the Kediri City Slaughterhouse are related to *Fasciola* sp. which infects dairy cattle from China. The distribution of *F. hepatica* and *F. gigantica* in Southeast Asia is influenced by livestock movements (Calvani and Šlapeta, 2021). The rapid development of livestock traffic in Southeast Asian countries is supported by the situation that the majority of Southeast Asian countries are developing countries (Quang *et al*., 2022). Developing countries are very active in livestock import-export activities, one of which is Indonesia (Rozaki, 2021). China is one of the countries that is quite active in importing livestock from Australia, Argentina and Brazil, which are endemic areas for *F. hepatica* (Hing *et al*., 2021).

The existence of international livestock trade can result in the introduction of various diseases, one of which is Fasciolosis (Nyindo and Lukambagire, 2015). Fasciolosis can be subclinical so that livestock infected with Fasciolosis often do not show symptoms and therefore often escape examination by a veterinarian (Stuen and Ersdal, 2022). This subclinical form of fasciolosis results in the escape of *Fasciola* sp. from endemic areas to non-endemic areas. Livestock traffic can carry *F. hepatica* from endemic areas to non-endemic areas, causing the two parasites to experience sympatric speciation (Garcia-Corredor *et al*., 2023).

Sympatric speciation is the existence of two or more types of a species in the same geographic conditions and time (Richards *et al*., 2019). This sympatric speciation drives hybridization. Hybridization of the two species of *Fasciola* sp. Often occurs in subtropical and warm areas (Haridwal *et al*., 2021). Hybridization between *F. hepatica* and *F. gigantica* has occurred in China and has been analyzed using nuclear DNA and mitochondrial DNA. Livestock import-export activities from China allow the introduction of *Fasciola* sp. to Indonesia.

Conclusion

In the PCR technique, the *nad4* marker can produce a gene amplicon of 752 bp. Phylogenetic tree analysis of *F. gigantica* which infects beef and dairy cattle in Kediri City Slaughterhouse Regional Technical Implementation Unit has a close relationship with *Fasciola* sp. which infects dairy cattle in Heilongjiang Province, China.

Acknowledgments

The authors thank Faculty of Veterinary Medicine, Universitas Airlangga and the entire staff of the Kediri Slaughterhouse.

Conflict of interest

The authors have declared no conflict of interest.

References

Alsulami, M.N., Mohamed, K., Wakid, M.H., Abdel-Gaber, R., Timsah, A.G., Al-Megrin, W.A.I., Khan, A., Elkholy, W.A., Abdelaal, K.A.A., Elshabrawy, H.A., El-Kady, A.M., 2023. Molecular Characterization of *Fasciola hepatica* in Sheep Based on DNA Sequences of Ribosomal ITS-1. Infect. Drug Resist. 16, 6661–6671. doi:10.2147/IDR.S421206.

Angles, R., Buchon, P., Valero, M.A., Bargues, M.D., Mas-Coma, S., 2022. One Health Action against Human Fascioliasis in the Bolivian Altiplano: Food, Water, Housing, Behavioural Traditions, Social Aspects, and Livestock Management Linked to Disease Transmission and Infection Sources. Int. J. Environ. Res. Public Health 19, 1120. doi:10.3390/ijerph19031120.

Anh, D.N., Anh, L.T., Tuan, L.Q., Bac, N.D., Tien, T.V., Phuong, V.T.B., Duong, T.T., Luc, N.K., Quang, N.B., 2018. Identification of *Fasciola* Species Isolates from Nghe An Province, Vietnam, Based on ITS1 Sequence of Ribosomal DNA Using a Simple PCR-RFLP Method. J. Parasitol. Res. 2018, 2958026. doi:10.1155/2018/2958026.

Ayaz, S., Ullah, R., AbdEl-Salam, N.M., Shams, S., Niaz, S., 2014. *Fasciola hepatica* in some Buffaloes and cattle by PCR and microscopy. Sci. World J. 2014, 462084. doi:10.1155/2014/462084.

Bankur, P.K., Nayak, A., Bhat, K., Bankur, R., Naik, R., Rajpoot, N., 2014. Comparison of culture and polymerase chain reaction techniques in the identification of *Tannerella forsythia* in periodontal health and disease, an *in vitro* study. J. Indian Soc. Periodontol. 18, 155–160. doi:10.4103/0972-124X.131312.

Beesley, N.J., Caminade, C., Charlier, J., Flynn, R.J., Hodgkinson, J.E., Martinez-Moreno, A., Martinez-Valladares, M., Perez, J., Rinaldi, L., Williams, D.J.L., 2018. *Fasciola* and fasciolosis in ruminants in Europe: Identifying research needs. Transbound. Emerg. Dis. 65, 199–216. doi:10.1111/tbed.12682.

Birlutiu, V., Birlutiu, R.M., 2023. The First Case of Human Hepatic Fasciolosis Presented as Hepatic Pseudotumor Histopathologically Diagnosed in Romania—A Case Report. Healthcare 11, 1451. doi:10.3390/healthcare11101451.

Bocek, M., Motyka, M., Kusy, D., Bocak, L., 2019. Genomic and Mitochondrial Data Identify Different Species Boundaries in Aposematically Polymorphic Eniclases Net-Winged Beetles (Coleoptera: Lycidae). Insects 10, 295. doi:10.3390/insects10090295.

Calvani, N.E.D., Šlapeta, J., 2021. *Fasciola* Species Introgression: Just a Fluke or Something More? Trends Parasitol. 37, 25–34. doi:10.1016/j.pt.2020.09.008.

Caravedo, M.A., Cabada, M.M., 2020. Human Fascioliasis: Current Epidemiological Status and Strategies for Diagnosis, Treatment, and Control. Res. Rep. Trop. Med. 11, 149–158. doi:10.2147/RRTM.S237461.

Cwiklinski, K., O'Neill, S.M., Donnelly, S., Dalton, J.P., 2016. A prospective view of animal and human Fasciolosis. Parasite Immunol. 38, 558–68. doi:10.1111/pim.12343.

da Costa, R.A., Corbellini, L.G., Castro-Janer, E., Riet-Correa, F., 2019. Evaluation of losses in carcasses of cattle naturally infected with *Fasciola hepatica*: effects on weight by age range and on carcass quality parameters. Int. J. Parasitol. 49, 867–872. doi:10.1016/j.ijpara.2019.06.005.

Dowling, D.K., Wolff, J.N., 2023. Evolutionary genetics of the mitochondrial genome: insights from Drosophila. Genetics 224, iyad036. doi:10.1093/genetics/iyad036.

Fox, N.J., White, P.C., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting impacts of climate change on *Fasciola hepatica* risk. PLoS One 6, e16126. doi:10.1371/journal.pone.0016126.

Gao, X., Wang, D., Zhang, Z., Quan, C., Zhou, S., Li, K., Li, Y., Zhao, S., Kong, X., Kulyar, M.F., Zeng, J., Li, J., 2022. Genetic Characterization and Phylogenetic Analysis of *Fasciola* Species Isolated From Yaks on Qinghai-Tibet Plateau, China. Front. Vet. Sci. 9, 824785. doi:10.3389/fvets.2022.824785.

Garcia-Corredor, D., Alvarado, M., Pulido-Medellín, M., Muñoz, M., Cruz-Saavedra, L., Hernández, C., Giraldo, J.C., Vásquez-Arteaga, L.R., Coronado, A.C.M., Ramírez, J.D., 2023. Molecular characterization of *Fasciola hepatica* in endemic regions of Colombia. Front. Vet. Sci. 10, 1171147. doi:10.3389/fvets.2023.1171147.

Haridwal, S., Malatji, M.P., Mukaratirwa, S., 2021. Morphological and molecular characterization of *Fasciola hepatica* and *Fasciola gigantica* phenotypes from co-endemic localities in Mpumalanga and KwaZulu-Natal provinces of South Africa. Food Waterborne Parasitol. 22, e00114. doi:10.1016/j.fawpar.2021.e00114.

Hing, S., Foster, S., Evans, D., 2021. Animal Welfare Risks in Live Cattle Export from Australia to China by Sea. Animals (Basel) 11, 2862. doi:10.3390/ani11102862

Lalor, R., Cwiklinski, K., Calvani, N.E.D., Dorey, A., Hamon, S., Corrales, J.L., Dalton, J.P., Verissimo, C.D.M., 2021. Pathogenicity and virulence of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* that cause the zoonosis Fasciolosis. Virulence 12, 2839–2867. doi:10.1080/21505594.2021.1996520.

Le, T.H., De, N.V., Agatsuma, T., Nguyen, T.G.T., Nguyen, Q.D., McManus, D.P., Blair, D., 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. Int. J. Parasitol. 38, 725–730. doi:10.1016/j.ijpara.2007.10.003.

Le, T.H., Nguyen, K.T., Nguyen, N.T., Doan, H.T., Le, X.T., Hoang, C.T., De, N.V., 2012. Development and evaluation of a single-step duplex PCR for simultaneous detection of *Fasciola hepatica* and *Fasciola gigantica* (family Fasciolidae, class Trematoda, phylum Platyhelminthes). J. Clin. Microbiol. 50, 2720–2726. doi:10.1128/JCM.00662-12.

McCoy, K.D., 2003. Sympatric speciation in parasites--what is sympatry? Trends Parasitol. 19, 400–404. doi:10.1016/s1471-4922(03)00194-6.

Nasreldin, N., Zaki, R.S., 2020. Biochemical and immunological investigation of fascioliasis in cattle in Egypt. Vet. World 13, 923–930. doi:10.14202/vetworld.2020.923-930.

Nyagura, I., Malatji, M.P., Mukaratirwa, S., 2022. Occurrence of *Fasciola* (Digenea: Fasciolidae) Species in Livestock, Wildlife and Humans, and the Geographical Distribution of Their Intermediate Hosts in South Africa-A Scoping Review. Front. Vet. Sci. 9, 935428. doi:10.3389/fvets.2022.935428.

Nyindo, M., Lukambagire, A.H., 2015. Fascioliasis: An Ongoing Zoonotic Trematode Infection. Biomed. Res. Int. 2015, 786195. doi:10.1155/2015/786195.

Oehm, A.W., Zablotski, Y., Hoedemaker, M., Campe, A., Strube, C., Jordan, D., Springer, A., Klawitter, M., Knubben-Schweizer, G., 2023. Associations of production characteristics with the on-farm presence of *Fasciola hepatica* in dairy cows vary across production levels and indicate differences between breeds. PLoS One 18, e0294601. doi:10.1371/journal.pone.0294601.

Opio, L.G., Abdelfattah, E.M., Terry, J., Odongo, S., Okello, E., 2021. Prevalence of Fascioliasis and Associated Economic Losses in Cattle Slaughtered at Lira Municipality Abattoir in Northern Uganda. Animals (Basel) 11, 681. doi:10.3390/ani11030681.

Quang, V.H., Levecke, B., Do Trung, D., Devleesschauwer, B., Lam, B.V.T., Polman, K., Callens, S., Dorny, P., Dermauw, V., 2022. *Fasciola* spp. in Southeast Asia: a systematic review and meta-analysis protocol. Syst. Rev. 11, 138. doi:10.1186/s13643-022-02013-3

Raina, O.K., Jacob, S.S., Sankar, M., Bhattacharya, D., Bandyopadyay, S., Varghese, A., Chamuah, J.K., Lalrinkima, H., 2015. Genetic characterization of *Fasciola gigantica* from different geographical regions of India by ribosomal DNA markers. J. Parasit. Dis. 39, 27–32. doi:10.1007/s12639-013-0276-7.

Richards, E.J., Servedio, M.R., Martin, C.H., 2019. Searching for Sympatric Speciation in the Genomic Era. Bioessays 41, e1900047. doi:10.1002/bies.201900047.

Rokni, M.B., Bozorgomid, A., Heydarian, P., Aryaeipour, M., 2018. Molecular Evidence of Human Fasciolosis Due to *Fasciola gigantica* in Iran: A Case Report. Iran J. Public Health 47, 750–754.

Rozaki, Z., 2021. Food security challenges and opportunities in indonesia post COVID-19. Adv. Food Secur. Sustain. 6, 119–68. doi:10.1016/bs.af2s.2021.07.002.

Saadatnia, A., Solhjoo, K., Davami, M.H., Raeghi, S., Abolghazi, A., 2022. Molecular Identification of *Fasciola* Isolated from the Liver of Meat Animals in Fars Province, Iran. J. Parasitol. Res. 2022, 4291230. doi:10.1155/2022/4291230.

Saijuntha, W., Tantrawatpan, C., Agatsuma, T., Wang, C., Intapan, P.M., Maleewong, W., Petney, T.N., 2018. Revealing genetic hybridization and DNA recombination of *Fasciola hepatica* and *Fasciola gigantica* in nuclear introns of the hybrid *Fasciola* flukes. Mol. Biochem. Parasitol. 223, 31–36. doi:10.1016/j.molbiopara.2018.06.004.

Silva, A.E.P., Freitas, C.C., Dutra, L.V., Molento, M.B., 2020. Correlation between climate data and land altitude for *Fasciola hepatica* infection in cattle in Santa Catarina, Brazil. Braz. J. Vet. Parasitol. 29, e008520. doi:10.1590/S1984-29612020065.

Stuen, S., Ersdal, C., 2022. Fasciolosis-An Increasing Challenge in the Sheep Industry. Animals (Basel) 12, 1491. doi:10.3390/ani12121491.

Tadayon, S., Sharifiyazdi, H., Moazeni, M., Divar, M.R., 2015. Molecular Differentiation of *Fasciola* Species and Characterization of Genetic Diversity of *F. gigantica* Using NADH Dehydrogenase I (ND1) Gene in the Endemic Areas of Iran. Iran J. Parasitol. 10, 9–18.

Thanasuwan, S., Tankrathok, A., 2021. Comparison of polymerase chain reaction and microscopy for the detection of *Fasciola* spp. in the fecal matter of domestic bovines in Kalasin Province, Thailand. Vet. World 14, 2878–2882. doi:10.14202/vetworld.2021.2878-2882.

Zhang, J.L., Si, H.F., Zhou, X.Z., Shang, X.F., Li, B., Zhang, J.Y., 2019a. High prevalence of fasciolosis and evaluation of the efficacy of anthelmintics against *Fasciola hepatica* in buffaloes in Guangxi, China. Int. J. Parasitol. Parasites Wildl. 8, 82–87. doi:10.1016/j.ijppaw.2018.12.010.

Zhang, X.X., Cwiklinski, K., Hu, R.S., Zheng, W.B., Sheng, Z.A., Zhang, F.K., Elsheikha, H.M., Dalton, J.P., Zhu, X.Q., 2019b. Complex and dynamic transcriptional changes allow the helminth *Fasciola gigantica* to adjust to its intermediate snail and definitive mammalian hosts. BMC Genomics 20, 729. doi:10.1186/s12864-019-6103-5.