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Survey on Prevalence, Risk Factors and Molecular Detection of Fasciola in Pakistan

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ABSTRACT

Fascioliasis is caused by the liver fluke *Fasciola hepatica*, poses significant threat to livestock and human health worldwide, particularly in Punjab, Pakistan. In this study, a comprehensive analysis of *Fasciola hepatica* infection was conducted in the livestock population of the Punjab region, focusing on the districts of Faisalabad, Lahore, and Bahawalpur. This study examined the liver and bile ducts of animals and collected live adult flukes for further analysis. Subsequently, DNA extraction was carried out and the cytochrome c oxidase subunit 1 gene of mitochondrial DNA was targeted using PCR amplification. The successful amplification of PCR products was confirmed through visualization on agarose gels. DNA sequencing was employed to characterize the *Fasciola hepatica* isolates. The findings of this study revealed that among the 1448 samples collected from buffalo, cattle, sheep, and goats, only 11.46% were infected with Fascioliasis. Buffalo exhibited the highest prevalence (14.16%), followed by cattle (12.81%), sheep (9.13%), and goats (8.12%). Among the breeds, Nilli-Ravi and H.F cross had the highest prevalence, while Khundi and Cholistani showed the lowest rates of infection. Fascioliasis was more common in females (14.52%) than in males (10.16%), with the 4-5 years age group having the highest prevalence (15.73%). This study provides crucial insights into the prevalence and distribution of Fascioliasis in the studied cattle population. Understanding the factors influencing infection rates can facilitate early detection and prompt treatment. However, further research is needed to develop effective control and prevention strategies for this significant livestock and public health concern in Punjab, Pakistan.

INTRODUCTION

Fascioliasis is a disease of economic importance caused by *Fasciola* species, with global distribution. *Fasciola* species include two major parasites, i.e., *Fasciola hepatica* and *Fasciola gigantica*. *F. hepatica* affects all domestic and wild ruminant species, which makes it a very important disease, concerning food security, as livestock contributes to a major portion of the gross domestic product (GDP) of Pakistan. The genus *Fasciola* belongs to the class Trematoda, family Fasciolidae, phylum Platyhelminthes of worms, having common name as flukes or common liver flukes (Admassu et al., 2015).

The history of *Fasciola* dates back to 1855 when Cobbold identified the specimens take from a giraffe in a travelling menagerie in England. In 1895, Railliet quotes in his book that, flukes extracted from cattle that were slaughtered at St. Louis, Senegal, and Africa (Railliet, 1895). The flukes were identified as *Fasciola hepatica angusta*. Blanchard identifies these flukes as *F. gigantica*. In a subsequent study LQOss reported the identification of liver flukes from cattle and buffalo in Egypt one year after the initial observation. These flukes were classified as *Distomum hepaticum var. degyptiaca* which is currently considered to be *F. gigantica*. The geographical distribution of *Fasciola* species is extensive. The occurrence of *Fasciola* has been reported in various continents including, Africa (Kenya, Senegal, Northern and Southern Rhodesia, Tanganyika), Asia (Assam, Ceylon, China, Formosa, India, Indo-China, Philippines), and Europe (Spain) (Alicata, 1938).

The disease has been primarily observed in cattle, buffalos, sheep, and goats and has the potential to infect other species including humans. The study indicates that the mature flukes primarily inhabit the bile duct, while immature flukes are typically found in liver parenchyma with infrequent occurrences in other organs. These worms exhibit flattened morphology and bear a great resemblance to the outline of laurel leaf. Adult flukes measure between 18-30mm in length and 4-13mm in width and exhibit coloration that ranges from dirty grey to brownish (Babalola *et al*., 2011).

Life cycle of the genus *Fasciola* starts from eggs excreted in feces of the definitive host and it necessitates an intermediate host, a snail that majorly lives in aquatic environment (Admassu *et al*., 2015). During the life cycle of liver fluke, they undergo many developmental stages in the snail host before ultimately adhering to the surrounding vegetation as cysts. The cysts are transferred to definitive host through grazing. In the gastrointestinal tract of the animal the cyst wall is dissolved and young fluke is emerged. Then it passes from small intestines to the hepatobiliary system of the animals. According to recent studies, the drifting phase of these flukes in liver ranges from 6-8 weeks, before they settle down in the bile duct, eventually. It takes 2.5-3 months from entering into the host to becoming adult and sexually matured. The impact of *Fasciola* spp. on livestock production is estimated to result in a loss of over 3 billion US dollars each year. Additionally, these parasites are known to cause zoonotic disease on a large scale (Rehman *et al*., 2020).

Cattle herds with *F. hepatica* infection rates of 25% or more suffer significant production losses. There is widespread occurrence of *F. hepatica* in dairy cattle across various countries. The economic impact of the acute fascioliasis is significant both directly and indirectly. This is due to parasites unique ability to consume blood up to 0.2-0.5ml per day, leading to anemia as well as impact on total protein levels especially albumin. Chronic fascioliasis on the other hand can result in reduced growth rate, milk production, wool production and feed conservation rates (Soulsby et al. 2000). Chronic fascioliasis results in prolonged inflammation of liver and bile duct often accompanied by decrease in overall health, digestive problems, ascites, edema, and production losses (Ther *et al*., 2001). Despite the significant economic impact, the development of therapeutic agents and vaccines has not been sufficient (Arifin *et al*., 2016). There is a lack of focus on practical and dependable diagnostic methods. The precision of diagnostic methods is crucial for successful control measures, especially in light of growing issue of drug resistance in *F. hepatica*. Conventional coprological techniques for analyzing fecal samples have been frequently utilized in research studies. However, their accuracy and dependability have been called into question (Caravedo *et al*., 2022). Despite the progress made in the developing immunological techniques their specificity and/or sensitivity can be limited. Advanced nucleic acid-based techniques have showed potential for the diagnosis of current infections. PCR based methods are particularly effective for specific diagnosis and genetic characterization of *Fasciola* species, surpassing conventional techniques (Rojas *et al*., 2014).

As far as could be ascertained, further research is required to check the parasite's evolution, drug resistance issue and linkage between these two factors in *Fasciola* species to improve and establish effective control measures against the disease. In view of the foregoing, the present study was conducted with following aims and objectives:

- To determine the prevalence of *Fasciola* species in cattle population of Punjab.
- To molecularly characterize *Fasciola* species from each other employing DNA extraction and PCR to check specie evolution.

MATERIALS AND METHODS

Study Location

Different agro-geoclimatic zones of Punjab were selected. These included abattoirs of Faisalabad, Lahore, and mainly Bahawalpur. These areas were selected on the basis of two major criteria's, first one being presence of snail population, and secondly feeding practices of these areas. Snail populations are abundant through the Bahawalpur region because of the availability of the ponds and grazing covers the main part of feeding regimen for the animals. There are reports of Fascioliasis in the upper Faisalabad regions including rural areas coinciding with Sargodha and Chiniot, and same goes for the rural areas of Lahore, nearing Pattoki and Sahiwal, due to larger populations of livestock.

Animal Demographics

Sampling was done for a period of 3 months from 15th of April to 25th of July. A questionnaire was prepared for the purpose of understanding prevalence in different species, breeds, sex, age, reason for slaughter, feeding practices, animal's body condition scoring and deworming practices, prior disease history, etc. of livestock, destined to be slaughtered in the abattoirs. Data was collected from Faisalabad region and from other two districts was provided by other veterinarians. This questionnaire helped in understanding population dynamics of clinical Fascioliasis and limiting factors in the treatment and control strategies of Fascioliasis. Thorough clinical examination was also conducted. In this clinical exam many criteria were examined. For example, body temperature was taken to check if the animal is suffering from any stress or infections. Second parameter that was noted was the dehydration status of the animal, to check if the animal is properly nourished or received a proper feed and water, prior slaughtering.

Furthermore, animal's body condition scoring (BCS) was done through internationally established standards of BCS in livestock animals. Next, capillary refill time (CRT) was also checked as it is an excellent measure of checking dehydration and anemia. Moving on, the next parameter was anemia status of the animal, anemia status of the animals' prior slaughter told us about the parasitic load and especially of those who utilize animal blood to feed on, and the hepatic health of the animal because the hepatitis liver abnormalities are often indicated through yellow conjunctival membrane. For this anemia status, animal's conjunctival membrane of both eyes was observed carefully, while opening the eyelids of the animal with the help of thumb and index finger and matched with the FAMACHA© eye color chart.

Sample Collection

After the slaughtering of the animals presented at the abattoirs, livers along with gall bladder were separated from the other offal's. Prior examination of the liver and bile duct necessary protocols were followed. A sterile surgical blade was used to incise the bile duct that runs on the transverse axis of the caudal side of the liver and ends into the gall bladder. The adult *Fasciola* worm or other developmental stages of the liver fluke were collected into a sterile plastic zipper bag with the help of thumb forceps. These zipper bags containing the liver fluke were carefully labelled with the sample ID number, date of collection, and location of the slaughter house from where it was collected. These bags were immediately shifted into icebox/ cool box containing ice pouches, to avoid deterioration/ putrefaction of the liver fluke.

Sample Dispatch and Transportation

Collected samples were preserved in 10% formalin solution and then packed in the cool box surrounded with ice pouches and dispatched through courier service and they were received in the Public Health/ Preventive Medicine Lab in the department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad. These samples were stored at refrigeration temperatures, until further processing.

Molecular Investigations

DNA Extraction: DNA extraction is a crucial process as it is necessary to obtain highly purified DNA fragments in order to study molecular characteristics such as organism's genetics, specie identification, number of haplotypes and variations/ mutations in the genes etc.

Procedure: Anterior portion of the liver fluke worm was cut and taken with the help of sterile scissors and weighed up to 30µg, to avoid ovaries and testes thus excluding hereditary DNA from the sample. The DNA extraction protocol for the specimen samples were according to the instructions of the manufacturer, by using the TIANamp genomic DNA extraction kit manufactured by TIANGEN BIOTECH.

Polymerase Chain Reaction

The identification of parasites was accomplished by conducting PCR amplification of specific genes from *F. hepatica*, using primers that targeted the cytochrome-c oxidase subunit 1 (*cox*1) gene of the mitochondrial DNA, which was specific to the genus. The process of PCR was employed to study isolated DNA fragments. PCR thermal cycler was used to generate the required large amounts of DNA for the research.

Recipe or the reaction mixture was prepared by mixing above ingredients inside PCR tubes and on the cool-pedi and then these tubes were run in the thermal cycler for the required time and amplicons were retrieved.

For the PCR process to work, the reaction mixture had to have four parts (reagents or chemicals). A sample of DNA or RNA from saliva, blood, feces, body fluids and waste, hair, a scraping of the skin, a parasite (as in this study), etc. Second, there are DNA primers, which are small pieces of single-stranded DNA that help make a matched set of bases. DNA taq polymerase is the third item on the list. It is an enzyme that helps make a second strand that is complementary of DNA. Lastly, a mix of adenine (A), thymidine (T), cytosine (C), and guanine (G) in a Nucleotide solution was used to make two copies of a DNA strand. It started with placing the DNA sample and the other chemical ingredients mentioned above into PCR tubes. The tubes were placed into the thermal cycler machine.

The solution went through 3 steps in the thermal cycler. These steps were denaturation, annealing, and extension.

Initial Denaturation: 94°C for 05 minutes	(1x)
Denaturation: 94°C for 30 seconds Annealing: 72°C for 45 seconds Extension: 72°C for 35 seconds	(35x)
Final Extension: 72°C for 10 minutes.	(1x)
Store: 4°C until further processing.	(∞)

Figure 1

Protocol for PCR Temperature Cycle, Number of Cycles, and Total Program Length.

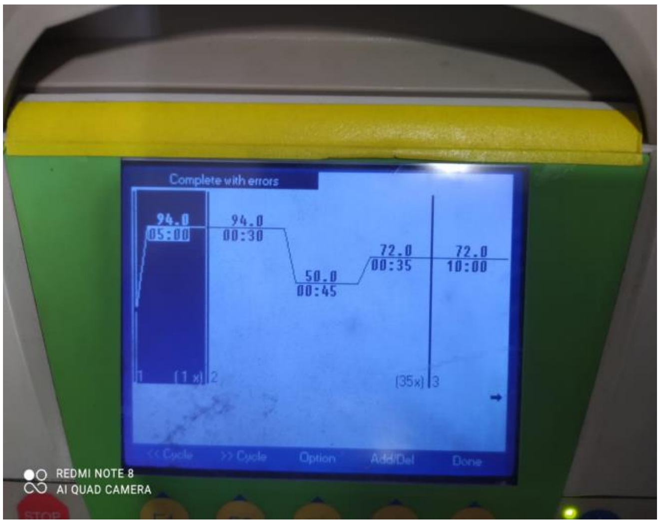
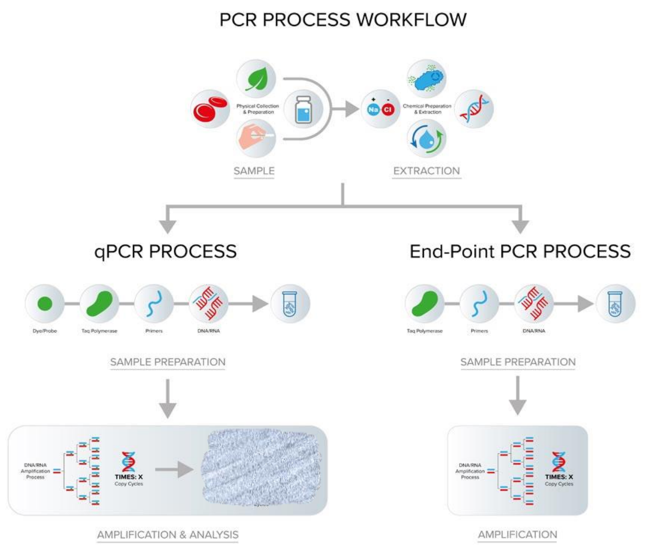


Figure 2

Pictorial Demonstration of PCR Process Workflow



Through this process, each of the individual strands of the initial specimen molecule were used to make a copy of a double-stranded DNA molecule. The entire procedure was repeated 35–40 times to make millions of copies of the DNA/RNA.

Gel Electrophoresis

Gel electrophoresis with 1.5% agarose gel was used to sort the expanded products. Gel electrophoresis is a way that DNA, RNA, or proteins are separated in the lab. An electrical current pushes the molecules of interest through a porous gel, with one side of the gel being charged positively and the other end being negatively charged. This makes molecules like DNA and RNA, which have a negative charge, move toward the positively charged end of the gel.

Procedure

It's a five-step process which goes as, preparing the samples, preparation of gel and the buffer, loading of samples, electrophoresis, visualized and documented bands.

Statistical Analysis

Vassar Stats and Statistix (version 10) was used to analyze all the prevalence data and its related drivers. P-values and chi-square values were computed. Using the WinPepi program, odds ratio and their confidence interval was determined. The 5% threshold of significance was applied for statistical significance.

RESULTS

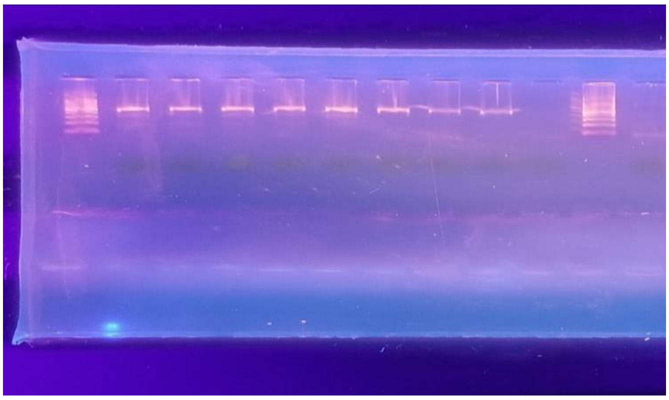
In this study, four different types of cattle were tested for Fascioliasis, a parasitic disease caused by liver flukes of the genus *Fasciola*. The study was done in the Punjab area, especially in the districts of Faisalabad, Lahore, and Bahawalpur. *Fasciola* infection was looked for in a total of 1448 samples taken from buffalo, cattle, sheep, and goats. Fascioliasis was found to be present in 166 of these cases.

Figure 3

Visualizing Bands on the Gel after Passing Electric Current

Figure 4

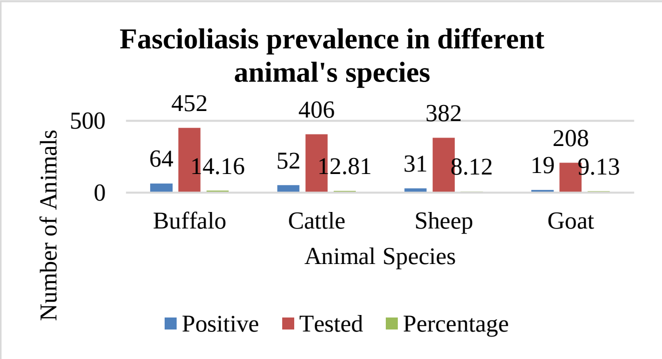
Visualizing Bands that Matched the DNA Ladder of 1000 Base Pair at 500 Base Pairs, on the Gel after Passing Electric Current



For each species, the rates of Fascioliasis were calculated. Fascioliasis was found in 14.16% of the animals that were tested. Of the 452 animals that were tested, 64 were positive for Fascioliasis. Cattle came in second with a frequency of 12.81 percent, meaning that 52 of the 406 animals tested were positive. Sheep had a lower rate of infection (9.13%), with 19 out of 208 animals tested positive. Fascioliasis was found in the fewest number of goats, 8.12%, or 31 out of 382 animals tested.

Figure 5

Graphical Representation of Prevalence of Ascioliasis in Different Animal's Species



Odds ratios (OR) were used to compare the chance of fascioliasis in each species to that of goats, which served as a reference group. If a buffalo tested positive for fascioliasis, the chances were statistically significantly higher (OR=1.74, 95% CI: 1.11-2.73) than if a goat did. Cattle were also more likely to test positive than goats (OR=1.58, 95% CI: 0.99–2.51), but the difference was not statistically significant. In the same way, sheep had a slightly higher chance of testing positive than goats (OR=1.13, 95% CI: 0.62-2.04), but the difference was not statistically significant. The study showed how common fascioliasis is in different types of cattle in the Punjab area. (Table 1)

Table 1

Showed Prevalence of Fascioliasis in Different Species of Ruminants

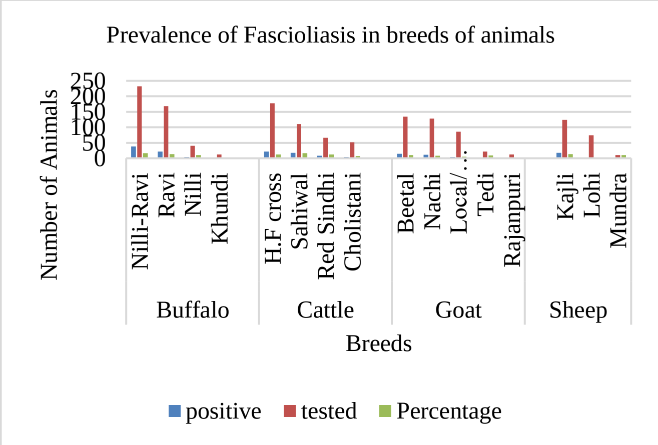
Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI
Animal's Identification Data						
Species	Buffalo	64/452	14.16% (10.95 - 17.37)	7.43 (0.0593)	1.74	1.11-2.73
	Cattle	52/406	12.81% (9.56 - 16.06)		1.58	0.99-2.51
	Sheep	19/208	9.13% (5.22 - 13.05)		1.13	0.62-2.04
	Goat	31/382	8.12% (5.38 - 10.85)		Ref.	Ref.

The number of animals with fascioliasis changed a lot between the different types of cattle. "Buffalo - Nilli-Ravi" had the highest frequency, at 16.38%, with 38 of 232 tested animals being positive. Statistically, this breed was more likely than goats to test positive for fascioliasis (OR = 4.14, 95% CI = 0.27–63.92, p = 0.3984). "Buffalo - Ravi" was next, with a frequency of 13.10%, meaning that 22 out of 168 animals tested were positive. Compared to goats, this breed also had a much higher chance of testing positive (OR=3.34, 95% CI: 0.21-52.27, p0.05). "Buffalo - Nilli" was found in 10% of the animals tested, or 4 out of the 40 animals. The chances of getting fascioliasis were much higher in this breed than in goats (OR = 2.78, 95% CI: 0.14–49.56, p 0.05). "Buffalo - Khundi" did not have any good cases, so its frequency was 0%.

Among cow breeds, "Cattle - H.F cross" was found in 16.36% of the animals tested, or 18 out of 110. The chances that this breed would test positive for fascioliasis were not much different from those of goats (OR=2.13, 95% CI: 0.69–6.54, p=0.5794). "Cattle - Sahiwal" had a frequency of 12.36%, which means that 22 of the 178 animals that were tested were positive. The chances that this breed would test positive for fascioliasis were not much different from those of goats (OR=1.61, 95% CI: 0.53–4.83, p=not given). "Cattle - Red Sindhi" was found in 12.12% of the animals tested, or 8 out of 66. The chances that this breed would test positive for fascioliasis were not much different from those of goats (OR=1.58, 95% CI: 0.45–5.47, p=not given). "Cattle - Cholistani" did not have any positive cases, so its frequency was 0%.

Figure 6

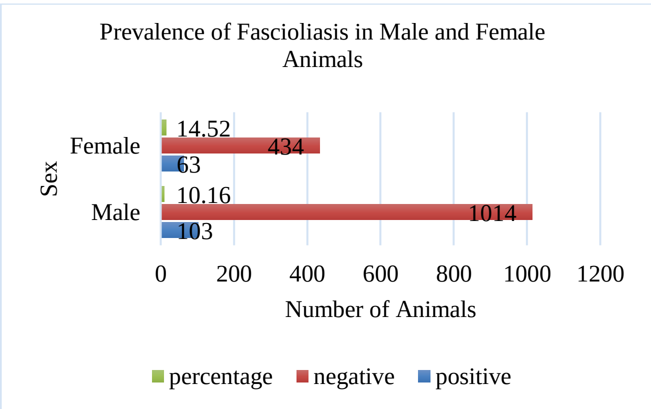
Graphical Representation of Prevalence of Fascioliasis in Different Breeds of Animals



For goat types, the "Goat - Beetal" fascioliasis was found in 14.5% of the 134 animals that were tested. The chances that this breed would test positive for fascioliasis were not significantly different from goats (OR=2.70, 95% CI: 0.17-42.97, p=0.5480). "Goat - Nachi" was found in 9.09% of the animals that were tested, or 2 out of 22. The chances that this breed would test positive for fascioliasis were not much different from those of goats (OR=2.78, 95% CI: 0.14-5.93, p=not given). "Goat - Local/Desi/Mix" was found in 8.59% of the animals that were tested, or 11 out of 128. The chances that this breed would test positive for fascioliasis were not much different from those of goats (OR=2.24, 95% CI: 0.14-36.12, p=not given). "Goat - Tedi" was found in 4.65% of the animals that were tested, or 4 out of 86. The chances that this breed would test positive for Fascioliasis were not much different from those of goats (OR=1.30, 95% CI: 0.07–23.05, p=not given). "Goat - Rajanpuri" did not have any good cases, so its frequency was 0%.

Figure 7

Graphical Representation of Prevalence of Fascioliasis in Male and Female Animals



In the study based on gender, fascioliasis was found in 14.52 percent of female animals, or 63 out of 434 animals that were tested. Female animals were much more likely to test positive for fascioliasis than male animals (OR=1.43, 95% CI: 1.02-1.99, p=0.05). A total of 103 of the 1014 male animals that were checked were positive, giving a frequency of 10.16 percent. (Table 2)

Table 2

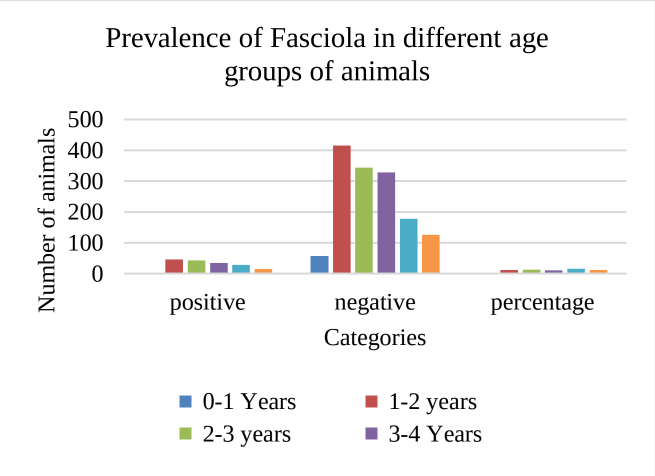
Illustrated Prevalence of Fascioliasis in Different Breeds of Animals and Showed Gender-Based Prevalence as Well

Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI	Variables
Breed	Buffalo	Nilli-Ravi	38/232	16.38% (11.62 - 21.14)	2.96 (0.3984)	4.14	0.27-63.92
		Ravi	22/168	13.10% (7.99 - 18.2)		3.34	0.21- 52.27
		Nilli	04/40	10.00% (0.7 - 19.3)		2.78	0.16- 49.56
		Kundi	00/12	0.00% (0 - 0)		Ref.	Ref.
	Cattle	H.F cross	18/110	16.36% (9.45 - 23.28)	1.97 (0.5794)	2.13	0.69-6.54
		Sahiwal	22/178	12.36% (7.52 - 17.19)		1.61	0.53-4.83
		Red Sindhi	08/66	12.12% (4.25 - 20)		1.58	0.45-5.47
		Cholistani	04/52	7.69% (0.45 - 14.94)		Ref.	Ref.
	Goat	Beetal	14/134	10.45% (5.27 - 15.63)	3.06 (0.5480)	2.70	0.17-42.97
		Nachi	02/22	9.09% (0 - 21.1)		2.78	0.14-55.93
		Local/ Desi/ Mix Breed	11/128	8.59% (3.74 - 13.45)		2.24	0.14-36.12
		Tedi	04/86	4.65% (0.2 - 9.1)		1.30	0.07-23.05
		Rajanpuri	00/12	0.00% (0 - 0)		Ref.	Ref.
	Sheep	Kajli	17/124	13.71% (7.66 - 19.76)	7.35 (0.0254)	10.15	1.34-76.78
		Lohi	01/10	10.00% (0 - 28.59)		7.40	0.46-118.31
		Mundra	01/74	1.35% (0 - 3.98)		Ref.	Ref.
Sex	Female		63/434	14.52% (11.2 - 17.83)	4.45 (0.0349)	1.43	1.02-1.99
	Male		103/1014	10.16% (8.3 - 12.02)		Ref.	Ref.

The study looked at how common fascioliasis is in different age groups. Different age groups of the animals studied showed big differences in the number of cases. With a frequency of 15.73%, 28 out of 178 tested animals in the age group "4-5 Years" were positive. The chances of having fascioliasis were significantly higher in this age group than in the reference group (0-1 Years) (OR=18.04, 95% CI: 1.12–293.03, p=0.0896)

Figure 8

Graphical Demonstration of Prevalence of Fasciola in Different Age Groups of Animals



The next age group, "2–3 Years," had a frequency of 12.50%, with 43 of the 344 animals tested positive. The chances of having Fascioliasis were much higher in this age group than in the reference group (0–1 years) (OR=14.27, 95% CI: 0.89–229.40, p=not given). Animals in the age group "More than 5 Years" had a frequency of 11.90%, with 15 out of 126 tested animals being positive. In this age group, the chances of testing positive for fascioliasis were much higher than in the reference group (0-1 Years) (OR=13.85, 95% CI: 0.82–227.78, p=not given).

In the "1-2 Years" age group, 46 of the 416 cats that were tested were positive, for a frequency of 11.06. The chances of having Fascioliasis were much higher in this age group than in the reference group (0–1 Years) (OR=12.62, 95% CI: 0.79–202.56, p=not given). In the "3-4 Years" age group, 10.37% of the animals were positive, or 34 out of 328 animals tested. The chances of having Fascioliasis were much higher in this age group than in the reference group (0–1 Years) (OR=11.87, 95% CI: 0.74–191.61, p=not given). There were no positive cases in the "0-1 Years" age group, so the frequency was 0.00%. (Table 3)

The rate of fascioliasis was very different depending on the reason for slaughtering. The animals that were slaughtered because they had "diseases/fractures/terminal illnesses" had the highest frequency, at 25.84%, with 46 of the 178 animals tested positive. In this group, the odds of testing positive for fascioliasis were much higher than in animals that were slaughtered because they were "old, infertile, or had a dead udder" (OR = 3.47, 95% CI: 2.10-5.73, p = 0.0000). Animals slaughtered for "meat selling" had a frequency of 10.29%, and the chances of testing positive were not significantly different from the reference group. Fascioliasis was found in 7.45% of animals that were killed because they were "old, infertile, or had a dead udder."

Table 3

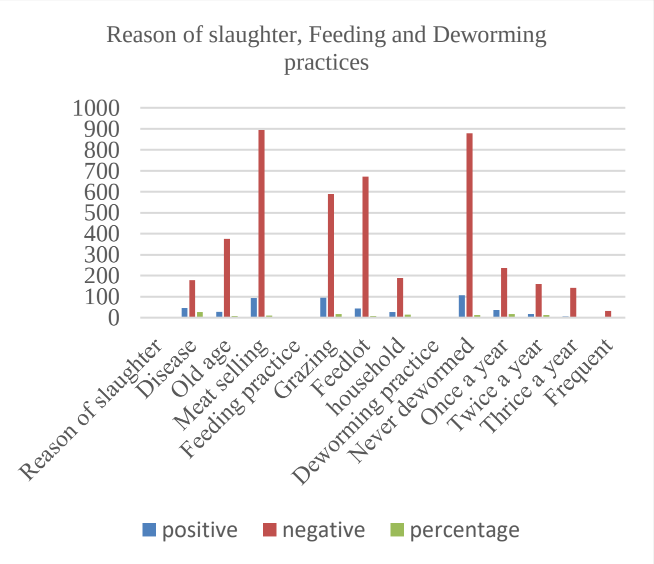
Showed the Prevalence of Fascioliasis in Different Age Groups of Animals

Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI
Age group	4-5 Years	28/178	15.73% (10.38 - 21.08)	9.53 (0.0896)	18.04	1.11-293.03
	2-3 Years	43/344	12.50% (9.01 - 15.99)		14.27	0.89-229.40
	More than 5 Years	15/126	11.90% (6.25 - 17.56)		13.85	0.83-229.78
	1-2 Years	46/416	11.06% (8.04 - 14.07)		12.62	0.79-202.56
	3-4 Years	34/328	10.37% (7.07 - 13.66)		11.87	0.74-191.61
	0-1 Years	00/56	0.00% (0 - 0)		Ref.	Ref.

The number of animals with fascioliasis also changed a lot based on how they were fed. The highest rate of infection was found in "Grazing" animals, at 16.33%, with 96 out of 588 tested animals being positive. The chances that an animal in this group would test positive for fascioliasis were much higher than in the "Household" group (OR=2.49, 95% CI: 1.72–3.62, p=0.0000). There were 13.83 percent of animals that tested positive in the "Feedlot" category, and the odds of that were significantly higher than in the "Household" category (OR=2.11, 95% CI: 1.27-3.52, p=not given). Fascioliasis was found in 6.5% of the animals that were fed in a "household" way.

Figure 9

Graphical Representation of Fascioliasis Prevalence according to Reason of Slaughter, Feeding and Deworming Practices



The frequency of deworming animals was linked to the number of cases of fascioliasis. Animals that were dewormed "Once a Year" had the highest frequency, at 16.10%, with 38 out of 236 checked animals being positive. In this group, the chances of testing positive for fascioliasis were much higher than in the "More frequent" group (OR=10.58, 95% CI: 0.66-169.09, p=0.0040). The number of animals that had never been dewormed was 12.07%, and the chances of testing positive were not significantly different from the reference group. The frequency of worms in animals dewormed "Twice a Year" was 10.63%, and the chances of testing positive were not significantly different from the reference group. Fascioliasis was found in only 3.52% of animals that were dewormed "Twice a Year."
The number of animals with fascioliasis was not linked to whether or not they had been sick before. Animals that had "Never" had a prior disease history had a frequency of 10.50%, and their chances of testing positive were not significantly different from animals that had "Yes Once/Twice" prior disease history. The results of the chi-square tests show that the rate of Fascioliasis in different groups within each variable changes significantly. (Table 4)

Table 4

Showed the Prevalence of Fascioliasis in Animals according to Reason of Slaughter, Feeding Practice, Deworming Practice, and Prior Disease History

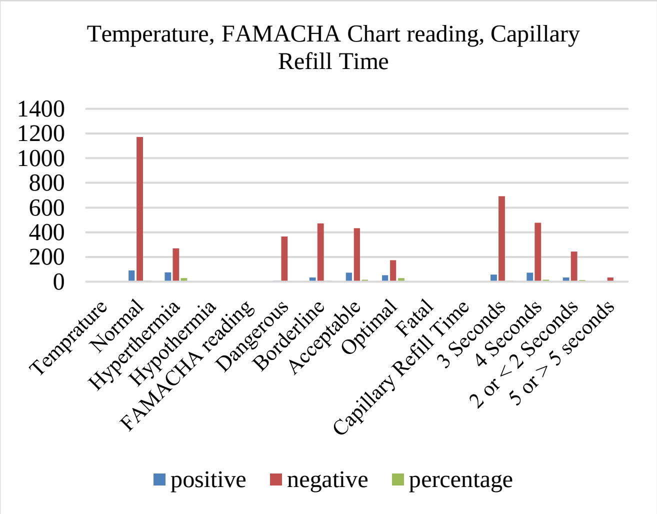
Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI
Reason for slaughter	Diseases/ Fractures/ Terminal illnesses	46/178	25.84% (19.41 - 32.27)	31.41 (0.0000)	3.47	2.10-5.73
	Meat selling	92/894	10.29% (8.3 - 12.28)		1.38	0.89-2.14
	Old age/ Infertility/ Dead udder	28/376	7.45% (4.79 - 10.1)		Ref.	Ref.
Feeding practices	Grazing	96/588	16.33% (13.34 - 19.31)	24.53 (0.0000)	2.49	1.72-3.62
	Feedlot	26/188	13.83% (8.9 - 18.76)		2.11	1.27-3.52
	Household	44/672	6.55% (4.68 - 8.42)		Ref.	Ref.
Deworming practice	Once a Year	38/236	16.10% (11.41 - 20.79)	15.37 (0.0040)	10.58	0.66-169.09
	Never	106/878	12.07% (9.92 - 14.23)		7.88	0.50-124.22
	Twice a Year	17/160	10.63% (5.85 - 15.4)		7.09	0.43-115.86
	Thrice a Year	05/142	3.52% (0.49 - 6.55)		2.51	0.14-44.62
	More frequent	00/32	0.00% (0 - 0)		Ref.	Ref.
Prior disease history	Frequently ill	47/218	21.56% (16.1 - 27.02)	19.46 (0.0001)	2.34	1.58-3.47
	Never	46/438	10.50% (7.63 - 13.37)		1.14	0.77-1.68
	Yes Once/ Twice	73/792	9.22% (7.2 - 11.23)		Ref.	Ref.

The frequency of fascioliasis was very different depending on the body temperature of the animal during the clinical test. Animals with "Hyperthermia" (an

abnormally high body temperature) had the highest frequency, at 28.15 percent, with 76 out of 270 tested animals being positive. Animals in this group were much more likely to test positive for fascioliasis than animals with "Hypothermia" (an abnormally low body temperature) (OR=3.68; 95% CI: 0.25–53.36; p=0.0000). 7.68% of animals had a "Normal" body temperature, and their chances of testing positive were not much different from those of animals with "Hypothermia."
The rate of fascioliasis was also linked to the numbers on the FAMACHA chart, which is used to measure anemia in animals. With a frequency of 29.31%, 51 out of 174 animals checked were positive for animals with a "Dangerous" reading, which means they have serious anemia. In this group, the chances of testing positive for fascioliasis were much higher than in the "Optimal" group (OR = 2.66, 95% CI: 0.19-36.91, p = 0.0000). Animals with "Borderline" and "Acceptable" readings had occurrence rates in the middle, while animals with "Fatal" readings (which mean death is close) did not have any good cases.

Figure 10

Graphical representation of Fascioliasis presence according to body temperature, FAMACHA chart reading, and Capillary refill time



The number of animals with fascioliasis also changed based on how long it took for the capillaries to refill during a clinical examination, which is a way to measure how well the blood flows. The animals with a refill time of "3 Seconds" had the highest frequency, at 15.06%, with 72 of the 478 animals tested positive. Animals in this group were more likely to test positive for fascioliasis (OR=2.56, 95% CI: 0.61-10.68, p=0.0074) than animals with "5 or > 5 Seconds" refill time. Animals with refill times of "4 Seconds" and "2 or 2 Seconds" had incidence rates in the middle. The results of the chi-square tests showed that the frequency of fascioliasis in different groups within each clinical screening variable changes significantly. (Table 5)

Table 5

Showed the Prevalence of Fascioliasis in Animals with Reference to Different Clinical Examinations Factors

Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI
Clinical Examination						
Temperature	Hyperthermia	76/270	28.15% (22.78 - 33.51)		3.68	0.25-53.36
	Normal	90/1172	7.68% (6.15 - 9.2)	65.45 (0.0000)	1.00	0.07-14.51
	Hypothermia	00/06	0.00% (0 - 0)		Ref.	Ref.
FAMACHA chart reading	Dangerous	51/174	29.31% (22.55 - 36.07)		2.66	0.19-36.91
	Borderline	73/432	16.90% (13.36 - 20.43)		1.53	0.11-21.11
	Acceptable	34/472	7.20% (4.87 - 9.54)	81.23 (0.0000)	0.66	0.05-9.17
	Optimal	08/366	2.19% (0.69 - 3.68)		0.21	0.01-3.11
	Fatal	00/04	0.00% (0 - 0)		Ref.	Ref.
Capillary refill time	3 Seconds	72/478	15.06% (11.86 - 18.27)		2.56	0.61-10.68
	4 Seconds	34/244	13.93% (9.59 - 18.28)	12.00 (0.0074)	2.37	0.56-10.11
	2 or < 2 Seconds	58/692	8.38% (6.32 - 10.45)		1.42	0.34-5.96
	5 or > 5 seconds	02/34	5.88% (0 - 13.79)		Ref.	Ref.

The rate of fascioliasis was very different depending on how long the skin tenting was done during the clinical test. The skin tent test time of "3 Seconds" showed the highest frequency at 14.94%, with 78 of the 522 animals tested positive. In this group, the chances of testing positive for fascioliasis were not statistically different from those of animals with a skin tent test time of "5 or > 5 seconds" (OR = 1.79, 95% CI: 0.55–5.87, p = 0.0135). The incidence rates of skin tent test times of "4 Seconds" and "2 or 2 Seconds" were in the middle.
The number of animals with fascioliasis varied depending on the heart rate that was recorded during the clinical exam. The number of infected animals with a "Normal" heart rate was 13.50%, or 37 out of 274 animals tested. Compared to animals with "Bradycardia" (an abnormally slow heart rate), the chances of testing positive for fascioliasis in this group were not statistically different (OR=3.42, 95% CI: 0.22-52.74, p=0.3055). The number of animals with "Tachycardia" (an abnormally high heart rate) was 11.10%, and the chances of testing positive did not vary statistically from the reference group.
The rate of breathing was a big factor in how common fascioliasis was found to be during a clinical test. The highest rate of infection was found in animals with a "Normal" breathing rate, which was 18.05%, or 74 out of 410 animals studied. When compared to animals with "Bradypnea" (an abnormally slow breathing rate), the chances of having Fascioliasis were much higher in this

group (OR=5.99, 95% CI: 0.39-92.81, p=0.0000). The number of animals with "Tachypnea" (an abnormally high breathing rate) was 9%, and the chances of testing positive did not vary statistically from the comparison group.

The results of the chi-square tests showed that the frequency of fascioliasis in different groups within each clinical screening variable changes significantly. But it's important to keep in mind that some risk ratios and p-values were not statistically significant. This means that the relationships seen in the data could be due to chance. (Table 6)

Table 6

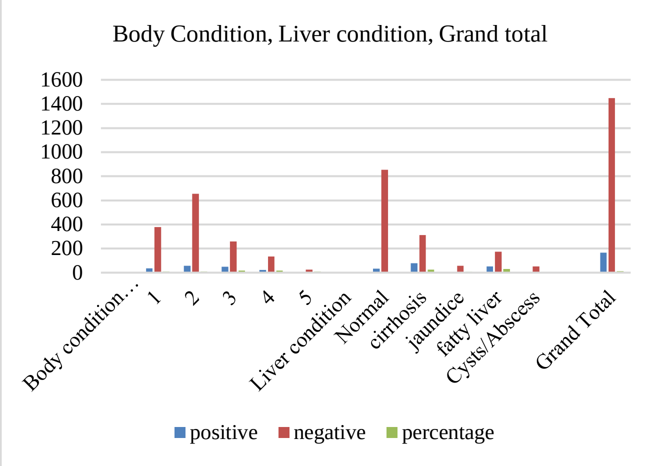
Showed the Fascioliasis Prevalence in Animals according to Skin Tent Test, Heart Rate, and Respiration Rate

Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI
Skin tent test time	3 Seconds	78/522	14.94% (11.88 - 18)	10.70 (0.0135)	1.79	0.55-5.87
	4 Seconds	23/166	13.86% (8.6 - 19.11)		1.66	0.48-5.75
	2 or < 2 Seconds	62/724	8.56% (6.53 - 10.6)		1.03	0.31-3.38
	5 or > 5 seconds	03/36	8.33% (0 - 17.36)		Ref.	Ref.
Heart rate	Normal	37/274	13.50% (9.46 - 17.55)	2.37 (0.3055)	3.42	0.22-52.74
	Tachycardia	129/1162	11.10% (9.3 - 12.91)		2.78	0.18-42.36
	Bradycardia	00/12	0.00% (0 - 0)		Ref.	Ref.
Respiration rate	Normal	74/410	18.05% (14.33 - 21.77)	19.93 (0.0000)	5.99	0.39-92.81
	Tachypnea	92/1022	9.00% (7.25 - 10.76)		2.99	0.19-46.13
	Bradypnea	00/16	0.00% (0 - 0)		Ref.	Ref.

The frequency of fascioliasis was very different between the animals with different body condition scores. Animals with a body condition score of "3" had the highest frequency (18.60%), with 48 out of 258 tested animals being positive. Animals in this group were more likely to test positive for fascioliasis (OR=2.23, 95% CI: 0.52-9.49, p=0.0019) than animals with a body condition score of "5". The frequency rates were in the middle for animals with body state scores of 4, 1, and 2.

Figure 11

Graphical Representation of Prevalence Data of Fascioliasis according to Body Condition, Liver Condition, and Grand Total



The frequency of fascioliasis also changed a lot based on how the liver looked when examined after slaughtering. The highest rate of "Liver Cirrhosis" was 29.89%, with 52 out of 174 tested animals being positive. Animals in this group had a much higher chance of testing positive for fascioliasis than animals with "Cysts/ Abscesses/ Granules" (OR=15.54, 95% CI: 2.14-113.03). Animals with "Jaundice/Inflamed (Rounded Borders)" also had a significantly higher frequency, at 25%, and the chances of testing positive were even higher than in the reference group (OR=13.00, 95% CI: 1.80-93.74, p=0.0000). Low incidence rates were seen in animals with "Fatty Liver" and "Normal" liver conditions.

The results of the chi-square tests showed that the rate of Fascioliasis is different in a big way between different categories of body condition scores and liver condition. (Table 7)

Table 7

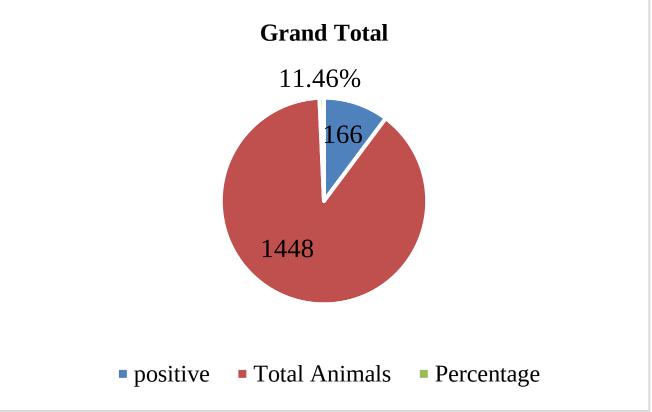
Showed Prevalence of Fascioliasis in Animals according to Their Body Condition Scoring and Liver Condition after the Slaughtering of Animals

Variables	Category	Positive/ Tested	Prevalence (95% CI)	Chi-square (p-value)	OR	95% CI
Body Condition Score	3	48/258	18.60% (13.86 - 23.35)	16.99 (0.0019)	2.23	0.52-9.49
	4	22/134	16.42% (10.15 - 22.69)		1.97	0.45-8.69
	1	36/378	9.52% (6.56 - 12.48)		1.14	0.27-4.90
	2	58/654	8.87% (6.69 - 11.05)		1.06	0.25-4.49
	5	02/24	8.33% (0 - 19.39)		Ref.	Ref.
Liver Condition	Liver Cirrhosis	52/174	29.89% (23.08 - 36.69)	128.09 (0.0000)	15.54	2.14-113.03
	Jaundice/ Inflamed (Rounded Borders)	78/312	25.00% (20.2 - 29.8)		13.00	1.80-93.74
	Fatty Liver	03/56	5.36% (0 - 11.25)		2.79	0.29-27.05
	Normal	32/854	3.75% (2.47 - 5.02)		1.95	0.27-14.27
	Cysts/ Abscesses/ Granules	01/52	1.92% (0 - 5.66)		Ref.	Ref.
Total Animals	1448	166/1448	11.46% (9.82-13.11)			

The study provided important information about how these factors were linked to the incidence of fascioliasis in the studied cattle group. Fascioliasis is a disease that affects animals and people all around the globe. If these links are known, the disease can be found and tracked early and quickly treated. But more research and understanding are needed to figure out what these links mean for controlling and preventing fascioliasis and what they mean for the future. Also, taking into account possible influencing factors and doing a multivariate analysis may give a more complete picture of the links between body condition scores, liver condition, and the number of farm animals with fascioliasis.

Figure 12

Graphical Representation of Total Number of Tested Animals, Number of Positive Animals, and Percentage Prevalence



This study provided us important information about how the disease spreads and how dangerous it is. But more statistical tests, like chi-square and logistic regression, would be needed to figure out how important these differences are and find out what might be putting Buffalo, Cattle, Sheep, and Goat at risk for Fascioliasis. This study helped us to learn more about how the disease spreads and gives us a starting point for making control and protection plans for the animals in the studied areas.

DISCUSSION

In this study, four different types of cattle were tested for Fascioliasis, a parasitic disease caused by liver flukes of the genus *Fasciola*. The study was done in the Punjab area, especially in the districts of Faisalabad, Lahore, and Bahawalpur. Fasciola infection was looked for in a total of 1448 samples taken from buffalo, cattle, sheep, and goats. Fascioliasis was found to be present in 166 of these cases, which is roughly around 11.46%. The study analyzed samples from buffalo, cattle, sheep, and goats, with buffalo had the highest prevalence (14.16%) followed by cattle (12.81%), sheep (8.12%), and goats (9.13%). Among different buffalo breeds, Nilli-Ravi had the highest prevalence (16.38%). Male animals had a prevalence of 10.16%, while females had a slightly higher prevalence of 14.52%. The age group of 4-5 years had the highest prevalence (15.73%).

Rehman *et al.*, (2020) described the progress of metabarcoding sequencing 'Trema biome' technique. Instead of using metabarcoding sequencing techniques or looking into the genetic variation and transmission patterns of the parasite. This study focused on the prevalence of the disease and associated factors, which looked at Fascioliasis prevalence among cattle, buffalo, sheep, and goats in the Punjab region. According to recent research, there is a lot of genetic variety among populations, and *F. gigantica* may be able to meiosis as well as self- and cross-breed inside certain hosts. The preponderance of single haplotypes in the majority of host species is attributed to a single introduction of *F. gigantica* infection, which also serves as a demonstration of how animal migrations can affect gene flow. This study focused on disease prevalence and epidemiological factors in a wider range of livestock species in the Punjab region, whereas previous studies went deeper into the genetic aspects and transmission dynamics of the parasite, which can be used to explain why this study's results differ from others.

Zafar *et al.*, (2019) studied the prevalence of Fascioliasis. Comparing both results, the Nilli-Ravi buffalo breed had the highest prevalence (16.38%) in this study, which focused on cattle, buffalo, sheep, and goats in the Punjab region. Buffalo had the highest prevalence (14.16%), followed by cattle (12.81%). Animals aged 4-5 years had the highest rates (15.73%), while females had a little higher frequency (14.52%) than males (10.16%). In contrast, previous study, which concentrated on certain tehsils in the area, discovered that sheep had a larger incidence than goats, with Tehsil Talla Gang having the greatest prevalence, followed by Chakwal, Kallar Kahar, and Choa Saiden Shah. Furthermore, the study showed how grazing methods affect prevalence, with animals that graze displaying greater rates. In addition, the teddy breed of goats exhibited a greater incidence of *F. hepatica* than the beetal and mixed breeds. These results highlight the species- and region-specific variations in fascioliasis prevalence, with both researches contributing significant knowledge to the disease's epidemiology in the Punjab cattle community.

Ahmad *et al*., (2017) conducted a study on prevalence of fascioliasis in small ruminants. Comparing both studies, this study was looked into the incidence of fascioliasis, which concentrated on cattle, buffalo, sheep, and goats in the Punjab region, but in this study didn't explicitly look into the Sargodha district or the effectiveness of fasciolicidal drugs. The present study, however, focused on the epidemiology of small ruminants fascioliasis in Punjab, Pakistan, across a specified period of time. Small ruminants had a greater overall incidence of fasciolosis (40.51%), with the most common species being *Fasciola hepatica* (35.64%) and *Fasciola gigantica* (8.21%), according to microscopic analysis of fecal samples. The study also discovered that females and adult animals had greater prevalence rates, and that malnourished animals had the highest illness incidence. The differences in results between this research, which covered a broader range of livestock in the Punjab region, and the new study focusing specifically on small ruminants in the Sargodha district, could be attributed to variations in the targeted animal species, geographical location, and the specific time frame of data collection, which can influence the epidemiology of Fascioliasis. These findings add regional specificity and valuable information about the *in-vivo* efficacy of fasciolicidal compounds, enhancing our understanding of the disease's economic significance in the Sargodha district's small ruminant population.

Ashraf *et al*., (2014) studied seasonal prevalence of *Fasciola hepatica* in Punjab. In comparison to this, this research included cattle, buffalo, sheep, and goats, discovered that seasonal variables had an impact on the incidence of fascioliasis, with the age range of 4-5 years showing the highest frequency (15.73%). The latest study, however, found a distinct seasonal trend, with fasciolosis frequency being highest in fall, followed by spring and winter, and lowest in summer. Furthermore, although the recent study found no appreciable changes in incidence among the five study locations, but this study found variances in prevalence among various animal species, genders, and age groups. These findings add to our understanding of the complexity of the illness in the Punjab by highlighting the dynamic character of fascioliasis epidemiology, which is impacted by seasonal and local variables.

Khan, U. J., & Maqbool, A. (2012) conducted research, which explored the infection rates in these distinct environments, citing infection rates of 22.6% in killed cattle, 17.5% in animals at livestock farms, 10.82% in veterinary facilities, and 8.76% in domestic cattle. Both studies found a seasonal trend in prevalence, with autumn having the greatest rates, followed by spring and winter, and summer having the lowest rates. In this study, however, which concentrated on cattle, buffalo, sheep, and goats in the Punjab region, was looked at the prevalence of fascioliasis in livestock under various management conditions without making a distinction between household cattle, livestock farms, slaughterhouses, or veterinary clinics. Additionally, my research revealed age and gender disparities in frequency, with older animals and females displaying greater rates, the new study also showed that older and male cattle had a higher infection rate. In light of varied management scenarios and seasonal fluctuations, this study findings together contribute to a deeper knowledge of fascioliasis epidemiology in the Punjab cattle community.

Shahzad *et al*., (2012) conducted research on prevalence of *Fasciola hepatica,* which employed fecal examination and PCR. The prevalence of Fascioliasis in this study, which concentrated on cattle, buffalo, sheep, and goats in the Punjab area, was 11.46% overall, with the buffalo showing the greatest frequency at 14.16% and the cattle at 12.81%. The prevalence rates for sheep and goats were 8.12% and 9.13%, respectively. Animals in the 4 to 5 years age group had the greatest frequency at 15.73%, while females exhibited a slightly higher prevalence of 14.52% compared to males at 10.16%. Their investigation, in contrast, was restricted to Lohi sheep and Beetal goats in the same area, and a reduced overall frequency was found using microscopic analysis and PCR. The study stressed that both species were equally likely to acquire *F. hepatica* infection. These variations in prevalence rates likely originate from differences in sample types, diagnostic techniques, and animal species studied, highlighting the complex nature of Fascioliasis epidemiology in the Punjab livestock population, with sheep having higher rates than goats.

Khan *et al*., (2009) carried out research on bovine fascioliasis, when compared with this research work which described the total prevalence was 11.46% of fascioliasis including cattle, buffalo, sheep, and goats in the Punjab region. It revealed a substantially higher overall frequency of 25.46%, with *Fasciola gigantica* (22.40%) being more prevalent than *F. hepatica* (3.06%), whereas this study detected greater prevalence in buffalo (14.16%) and cattle (12.81%). This research study also observed changes in frequency between animal species, gender, and age groups, but the latest study discovered no appreciable differences in prevalence across age groups or sexes in buffaloes or cattle (30.50% vs. 20.42%). Both investigations demonstrated the influence of environmental variables on the occurrence of fascioliasis, including grazing patterns, mixed farming, and water supplies. The current study also showed a favorable economic effect, with fasciolicides-treated animals producing more milk and having better fat composition, yielding a cost-benefit ratio of 3.9. These findings highlight the intricacy and geographical differences in fascioliasis epidemiology as well as its possible economic repercussions on the cattle population in Punjab.

Tasawar *et al*., (2007) conducted research on fascioliasis in goats, keeping both studies in context; it is evident that in current study the prevalence of fascioliasis in the Punjab region's including cattle, buffalo, sheep, and goats had variations in the prevalence rates as compared to previous study. This research discovered a lower overall prevalence of 9.13% in goats, compared to their report of a prevalence of 28.75%. Additionally, the prevalence rates for different goat breeds differed between the two studies, with teddy goats having a greater frequency (42.1%) in previous study compared to current investigation, which lacked breed-specific prevalence data. Additionally, there were discrepancies between the two studies in the prevalence of fascioliasis according to age. According to Tasawar *et al*., the illness was more prevalent in goats between the ages of 13 and 24 months (35.71%), however in current study, the highest incidence was identified in the age bracket of 4-5 years (15.73%) across all species. These disparities highlight the impact of regional characteristics and study methodology on findings on the prevalence of fascioliasis and can be attributable to differences in geographic areas, sample sizes, and the particular breeds or age groups evaluated.

Iqbal *et al*., (2007) conducted research on prevalence of fascioliasis in cattle, buffalo, sheep, and goats in the Punjab region which showed some parallels with the current study. Both studies noted the presence of *F. hepatica* in agricultural animals in the Punjab region. However, current research did not distinguish precisely between regions that produced milk and those that did not, which would account for any variations in the prevalence rates that were found. In addition, while both studies recognized the possibility of anthelmintic therapy as a way of illness management, the primary focus of present study was on prevalence rates and epidemiological variables rather than therapeutic approaches. The importance of contextual factors is highlighted when interpreting results related to fascioliasis prevalence and control in the Punjab region. Differences in results may result from variations in the specific geographical locations or sample sizes taken into account, as well as differences in management practices.

Maqbool *et al*., (2002) did a study of fasciolosis in different parts of Punjab. Comparing current findings to their research on fascioliasis prevalence in cattle, buffalo, sheep, and goats in the Punjab region, there are both parallels and variances. In both investigations, fascioliasis was discovered in buffaloes living in households, farms, and slaughterhouses, among other places. The largest prevalence was seen on cattle ranches, according to Maqbool *et al.* who also discovered a seasonal pattern, with the maximum incidence occurring in the fall, spring, winter, and summer. In contrast, present study did not include seasonality or location-based variances, which may be related to different sample sizes, study sites, or management strategies. The variations in Fascioliasis prevalence findings in the Punjab area are likely influenced by these environmental variables.

CONCLUSION

In conclusion, this study, which analyzed 1448 livestock samples from Faisalabad, Lahore, and Bahawalpur in the Punjab area, explored light on the prevalence and factors associated with fascioliasis in farm animals. The research encompassed various animal types, breeds, genders, and age groups, revealing significant variations in disease frequency. Buffalo had the highest prevalence, followed by cattle, sheep, and goats, with specific breeds exhibiting higher susceptibility. The study also found a slightly higher prevalence in females than males and identified the age group of 4-5 years as the most susceptible.

These findings provide valuable insights into the diversity of the parasite in the region and underscore the need for targeted disease control measures. Moving forward, it is crucial to conduct longitudinal studies and multivariate analyses to better understand the risk factors and transmission dynamics of fascioliasis. This research lays the foundation for effective disease control and prevention strategies, emphasizing the importance of early detection and swift action to safeguard the health and productivity of farm animals in the Punjab area and beyond. The insights gained can inform policymakers, veterinarians, and livestock owners in developing tailored plans to reduce fascioliasis cases and promote sustainable livestock management practices.

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