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Research Article

Molecular diagnosis and therapeutic trials against bovine fasciolosis in and around Okara

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Abstract: Bovine fasciolosis, caused by *Fasciola hepatica* and *Fasciola gigantic*a, is a globally significant parasitic disease in cattle, resulting in substantial economic losses to the livestock industry. In Pakistan, particularly in the Okara region, the disease remains endemic due to environmental conditions conducive to parasite transmission. This study aimed to determine the prevalence of bovine fasciolosis in Okara using conventional and molecular diagnostic techniques and to assess the efficacy of various therapeutic interventions. A total of 348 samples (174 buffaloes and 174 cattle) were collected via simple random sampling, primarily from herds raised on natural grazing and seasonal green fodder. Fasciola eggs were isolated from fecal and bile samples using a standard washing-sieving method, followed by DNA extraction. Polymerase chain reaction (PCR) was performed under standardized conditions, with amplicons analyzed via agarose gel electrophoresis. Microscopic examination revealed a 14.36% (25/174) positivity rate in cattle, whereas PCR detected a higher prevalence of 16.67% (29/174) for F. hepatica, confirming PCR as a more sensitive and accurate diagnostic tool. This study enhances the understanding of fasciolosis in endemic areas and supports the development of improved diagnostic and treatment strategies for bovine fasciolosis.

Keywords: Bovine fasciolosis; molecular diagnostics; PCR; anthelmintic resistance; therapeutic trials

Received: 28 March 2025 Accepted: 7 May 2025 Published: 15 May 2025

Cite this Article: Ejaz, H. K., & Siddique, M. I. (2025). Molecular diagnosis and therapeutic trials against bovine fasciolosis in and around Okara. *Animal BioSphere*, *1 (1), 1-7.*

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Introduction

Bovine fasciolosis is a significant parasitic disease caused by *Fasciola hepatica* and *Fasciola gigantica*, which primarily affect the liver of cattle, leading to substantial economic losses in livestock production [1,2]. The disease is prevalent in tropical and subtropical regions, including Pakistan, where the presence of suitable intermediate hosts, such as freshwater snails, facilitates its transmission. Fasciolosis results in reduced productivity due to liver damage, decreased milk yield, poor weight gain, and increased susceptibility to secondary infections [3,4]. Additionally, acute illness symptoms, such as hemorrhagic hepatitis, anemia, and even mortality, can develop from serious infections. The importance of developing effective diagnostic and treatment solutions for reducing bovine fasciolosis cannot be overstated, given its economic and veterinary implications [5]. The genus Fasciola is found all over the globe, and it causes a parasitic liver illness in both wild and farmed ruminants. Economic losses resulting from fasciolosis include deaths, miscarriages, slowed growth, lower milk and meat output, condemnation of tainted liver and starved carcasses, and treatment costs for ill animals[6]. Factors like climate trends, grazing behavior, and the presence of disease-carrying snails affect the worldwide spread of bovine fasciolosis. Particularly in the Okara region, which is well-known for its large cow ranching, the disease poses a continual danger to the welfare and output of cattle in Pakistan [7]. As seen by the different prevalence rates reported in research, seasonal variations exert a major influence on the spread of illnesses. Losses suffered at abattoir liver condemnation, worse feed conversion efficiency, and more veterinary expenditures connected to treatment and control initiatives all help to determine the financial effect of fasciolosis. Good disease control depends on a correct and quick fasciolosis diagnosis. Among the more conventional approaches of diagnosis are sedimentation techniques on faecal eggs, postmortem investigations, and clinical tests [8]. Early or chronic infections with their modest parasite load and intermittent egg shedding render these treatments less than perfect. Advances in molecular diagnostics, more exact and sensitive approaches for spotting Fasciola infections have been created. Tests based on polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), or enzyme-linked immunosorbent assays (ELISA) might help one more precisely identify the early phases of fasciolosis[9,10]. These techniques enable faster detection of parasite DNA or circulating antigens in serum, blood, or faeces samples, therefore facilitating improved disease management strategies and faster response. Treating bovine fasciolosis mostly with triclobenzale, albendazole,

and closantel is anthelmintic action [11]. Against all kinds of flukes, triclobenzazole has demonstrated the best results. Still, fresh data on triclabendazole resistance emphasises the need of combination treatment and various alternative therapies [12]. Among the various treatment opportunities that have lately been explored include anthelmintic derived from botanicals, pharmacological formulations based on nanotechnology, and immunotherapeutic approaches. To develop long-term control plans in endemic regions like as Okara, it is imperative to carry out clinical studies to evaluate the efficacy of various treatment approaches [13,14]. The purpose of this research is to evaluate the effectiveness of various anthelmintic medications and alternative treatment approaches for bovine fasciolosis in the Okara area, as well as to integrate molecular diagnostic methods [15].

Materials and Methods

The present study investigated Fasciola infection (fasciolosis) in cattle and buffaloes in the Okara district of Punjab, Pakistan. Samples were processed at the Livestock Production Research Institute (LPRI) laboratory in Bahadurnagar, Okara. A total of 348 samples (174 buffaloes and 174 cattle) were collected using a simple random sampling technique, irrespective of age and sex. The animals selected were primarily from herds reared on natural grazing and seasonal green fodder. Sampling was based on disease history, clinical signs, postmortem lesions (in deceased animals), and suspected fasciolosis cases. Fecal samples (10 g each) were collected directly from the rectum or immediately after defecation. Each sample was labeled with the animal's identification number, collection date, and location, then transported to the laboratory at 4°C for further analysis, following the standard protocol described by Urquhart et al. (1996).

DNA Extraction

For the isolation of Fasciola eggs, fecal and bile samples were processed using a standardized washing-sieving technique, following the protocol described by Suhardono et al. (2006) with minor modifications. Briefly, 3 g of each fecal sample was homogenized in a 1% solution of commercial detergent (Surf Excel®) as a suspending fluid. The mixture was sequentially filtered through stacked sieves with apertures of 1 mm, 450 µm, and 266 µm to remove fibrous debris while retaining parasite eggs. The resulting sediment was collected for further DNA extraction [16,17]. The recovered eggs from fecal and bile samples were resuspended in physiological saline to obtain a pooled egg suspension with a final volume of 100 mL. The suspension was vortexed with glass beads overnight to disrupt the egg shells, following the protocol described by Ahmed and Khosa (2010). After vortexing, the mixture was centrifuged at 15,000 rpm for 15 minutes using a Sorvall® Ultraspeed Centrifuge (Thermo, USA). The supernatant was discarded, and 300 µL of the resulting sediment was used for total genomic DNA extraction. DNA extraction was performed using a commercial kit (Fermentas, Cat. #K0512) in accordance with the manufacturer's instructions. The extracted DNA was then stored at -20°C until further molecular analysis [12,18].

Table 1: The following primers were used in the present study.

Primer	Sequence	Amplicons (bp)	Annealing Temp	Source
DSJF DSJ3	ATATTGCGGCCATGGGTTAG CCAATGACAAAGTGACAGCG	300	600C	Ai et al. (2010)

The PCR amplification reactions were performed in a final volume of 25 µL containing 5 µL of DNA template, 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, #K1081), 1 µL of 25 mM $MgCl_2$ (Thermo Scientific), 2 µL each of forward and reverse primers, 2 µL of nuclease-free water (Fermentas, #R0581), and 0.5 µL of Taq DNA polymerase. The reaction mixtures were prepared under sterile conditions and subjected to thermal cycling using optimized amplification parameters. The resulting PCR products were subsequently analyzed through agarose gel electrophoresis to confirm successful amplification [19,20]. The PCR was carried out with following conditions i.e. initial denaturation at 95°C for 4 min and then for each of 30 cycles, the denaturation at 95°C for 1 min, annealing at 60 °C for 1 min and 3rd stage extension at 72 °C for 2 min. PCR cycles were followed by 10 min of final extension at 72 °C [21]. And at the end, final holding temperature was 4 °C until the PCR tubes were taken out of thermal cycler and placed in refrigerator or run on agarose gel Ai et al. (2010).

Results

The PCR analysis using DSJF/DSJ3 primers successfully amplified approximately 300-bp fragments in all *F. hepatica*-positive samples from both cattle and buffaloes, as demonstrated in Figure 1. Diagnostic results revealed that among 174 cattle samples (87 fecal and 87 bile), microscopic examination detected 25 positive cases (14.36%), while PCR identified 29 positives (16.67%). Similarly, examination of 174 buffalo samples (87 fecal and 87 bile) showed 30 microscopy-positive cases (17.24%), with PCR detecting a higher prevalence of 37 positive samples (21.26%). The complete results of Fasciola-positive samples from both fecal and bile specimens are presented in Table 2, confirming the superior sensitivity of PCR over conventional microscopic examination for fasciolosis diagnosis.

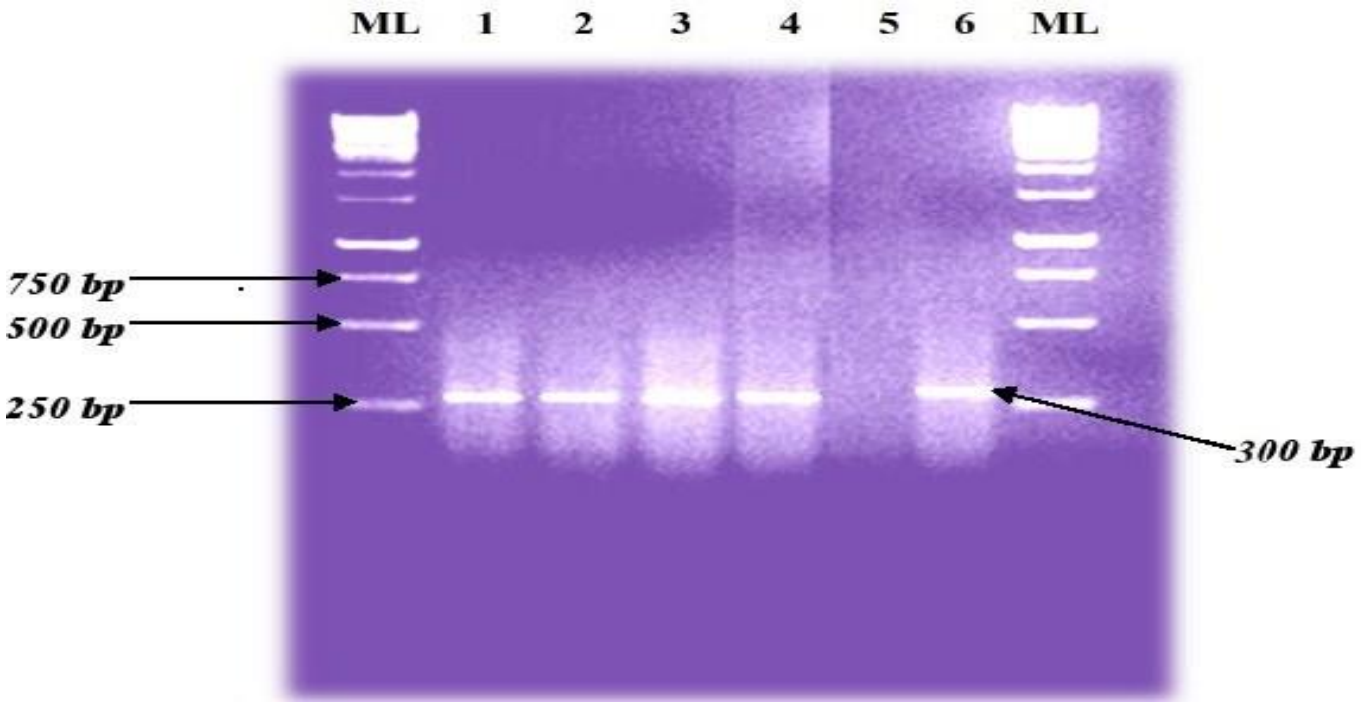

Figure 1. Agarose gel electrophoresis of PCR-amplified DNA from *F. hepatica* isolates in cattle and buffaloes using species-specific primers (300 bp target). Lane M represents the molecular weight ladder. Lanes 1 and 2 display amplified products from cattle fecal and bile isolates, respectively, while lanes 3 and 4 correspond to buffalo fecal and bile isolates. Lane 5 shows the negative control, and lane 6 the positive control. The clear 300-bp bands in lanes 1–4 confirm successful amplification of *F. hepatica* DNA from both host species and sample types, with controls validating assay specificity.

Table 2. Comparative diagnosis of fasciolosis in cattle and buffaloes using microscopic examination and PCR analysis

Animal Species	Sample tested	Positive samples			
		Microscopic	% age	PCR	% age
Cattle	174	25	14.36	29	16.67
Buffaloes	174	30	17.24	37	21.26

Molecular Diagnosis

The comparative analysis of *F. hepatica* detection methods revealed significant differences in diagnostic sensitivity between microscopy and PCR. In cattle, microscopic examination identified 25 positive samples (14.36%), whereas PCR detected 29 infections (16.67%). Similarly, in buffaloes, microscopy yielded 30 positive cases (17.24%), while PCR confirmed a higher prevalence of 37 infections (21.26%) (Figure 2). These results demonstrate that PCR consistently outperformed conventional microscopy in detecting fasciolosis, likely due to its ability to identify low-intensity or early-stage infections that may be missed by fecal egg counts. The disparity in detection rates underscores the importance of molecular tools for accurate disease surveillance, particularly in endemic regions where subclinical infections are common.

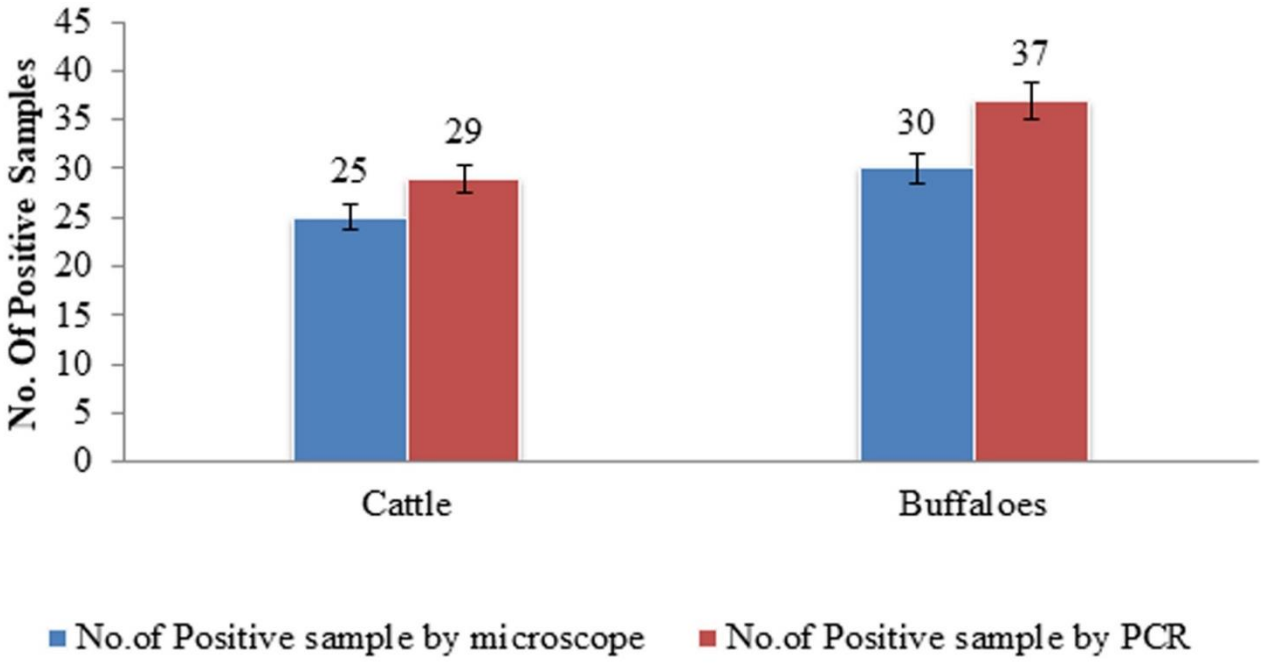

Figure 2. Comparison of *F. hepatica*-positive samples detected by microscopy versus PCR in cattle and buffaloes. Microscopic examination identified 25 positives in cattle and 30 in buffaloes (blue bars), while PCR detected 29 and 37 positives, respectively (orange bars). The higher positivity rates with PCR highlight its enhanced sensitivity for fasciolosis diagnosis.

Therapeutic interventions against fasciolosis

The efficacy of therapeutic interventions against fasciolosis was evaluated over a 14-day period, with distinct response patterns observed between treatment groups (Figure 3). On Day 3, Group A demonstrated a 54.5% reduction in parasite burden, increasing to 57.14% by Day 7 and reaching 73.33% by Day 14. In contrast, Group B showed a more gradual decline, with efficacy rates of 41.66% (Day 3), 29.41% (Day 7), and 68% (Day 14). Notably, while Group A achieved higher early-phase efficacy, Group B exhibited a significant late-phase improvement, suggesting differential time-dependent drug actions.

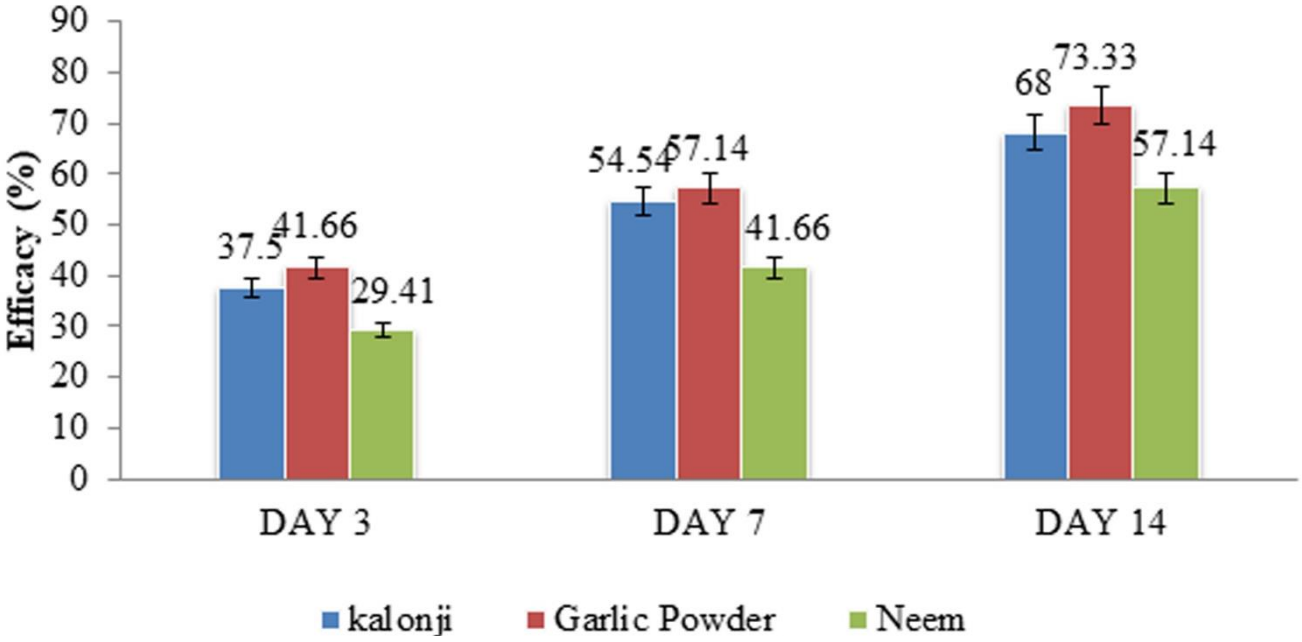

Figure 3. Time-course evaluation of therapeutic efficacy against fasciolosis. Bars represent percentage reduction in parasite burden for Group A (blue) and Group B (orange) at Days 3, 7, and 14 post-treatment. Group A showed rapid early-phase efficacy (73.33% by Day 14), whereas Group B demonstrated delayed but substantial late-phase response (68% by Day 14).

Discussion

Accurate identification and genetic characterization of parasites are fundamental to understanding disease epidemiology and improving diagnostic, treatment, and control strategies (Gasser & Newton, 2000; Gasser & Chilton, 2001; Gasser et al., 2002). In the present study, molecular diagnosis of *F. hepatica* infection was conducted using both fecal and bile samples from cattle and buffaloes, revealing critical insights into detection efficacy. Microscopic examination of 174 cattle samples identified 25 positive cases (14.36%), while PCR detected a higher prevalence of 29 infections (16.67%). This discrepancy aligns with findings by Maqbool et al. (2012), who reported variable infection rates (8.76%–22.6%) in cattle across different environments using coprological methods. The superior sensitivity of PCR observed in our study highlights its diagnostic advantage, particularly in detecting low-intensity infections that may be missed by conventional microscopy. The higher detection rate achieved through PCR underscores its potential as a reliable tool for fasciolosis surveillance. This enhanced diagnostic capability is critical for implementing timely and targeted control measures, ultimately reducing the economic and health impacts of the disease in livestock populations. Future studies could further explore the cost-effectiveness and field applicability of PCR-based diagnostics to optimize their use in endemic regions [22]. The infection rate was detected on the basis of identification of eggs in fecal samples by direct smear, floatation and sedimentation techniques Urquhart et al. (2001). Similarly in buffaloes out of 174 (87 fecal; 87 bile) samples 30 (17.24%) samples were positive by microscopic examination and 37(21.26%) samples were positive by PCR test. Maqbool et al. (2002) found that in buffaloes the infection rate of fasciolosis was 25.59%, 26.16%, 13.7% and 10.5%, respectively in slaughter house, at livestock farms, veterinary hospitals and in household buffaloes [23]. While this study employed PCR as a molecular diagnostic tool for *F. hepatica* identification - demonstrating superior reliability, sensitivity, and accuracy compared to conventional methods - the observed differences in prevalence rates between diagnostic techniques were not statistically significant (Cattle: $p=0.554$; Buffalo: $p=0.456$). This finding suggests that while PCR offers technical advantages in detection capability, its diagnostic superiority in field conditions may require larger sample sizes for conclusive validation. Historically, fasciolosis diagnosis in the region relied solely on clinical symptoms and microscopic examination of fecal samples, lacking the precision of modern molecular techniques. Our study represents a significant methodological advancement by implementing PCR-based detection in both fecal and bile samples from cattle and buffaloes. This approach not only confirms the presence of infection but also enables genetic characterization of parasites, which is crucial for understanding strain variations and potential drug resistance patterns [24]. Our findings demonstrate that PCR detected higher prevalence rates of fasciolosis in both cattle (16.67%) and buffaloes (21.26%) compared to microscopic examination (14.36% and 17.24% respectively), confirming PCR's superior sensitivity as a diagnostic tool. While these differences were not statistically significant ($p>0.05$), the technical advantages of molecular detection are evident, particularly for low-intensity infections. Notably, our protocol successfully amplified the characteristic 300bp fragment using *F. hepatica*-specific primers (DSJF/DSJ3), while showing no cross-reactivity with *F. gigantica* primers (DSJF/DSJ4) or negative controls - validating the assay's specificity. This study represents the first molecular characterization of *F. hepatica* in cattle from Pakistan's Okara district, employing a modified PCR protocol with optimized $MgCl_2$ (2µl) and Taq DNA polymerase (0.5µl) concentrations. Our results align with previous work by Ai et al. (2010) [25], who similarly established PCR's diagnostic superiority for Fasciola species differentiation. The consistent 300bp amplification pattern across both cattle and buffalo samples (Figure 1) further supports the reliability of this molecular approach, as also observed in small ruminants [26].

Conclusions

Bovine fasciolosis remains a significant health and economic concern for the livestock industry, particularly in endemic regions such as Okara. The disease, caused by *F. hepatica* and *F. gigantica*, leads to substantial productivity losses, including reduced milk yield, impaired growth, and increased mortality in infected cattle. Triclabendazole, albendazole, and closantel are among the anthelmintic drugs used mostly in bovine fasciolosis therapy. The development of drug-resistant Fasciola strains is a major obstacle that emphasises the necessity of substitute therapy approaches. Promising results from recent investigations on herbal anthelmintics, nanoparticle-based drug delivery technologies, and immunotherapeutic approaches indicate possible routes for sustained control of fasciolosis. Evaluating the efficacy, safety, and practicality of these new medicines depends on doing therapeutic trials in endemic areas like Okara. This work emphasises the need of integrating rigorous therapy trials with cutting-edge diagnostic tools to develop efficient and long-lasting management plans for bovine fasciolosis. The results improve knowledge of illness frequency, hone early detection strategies, and help to create fresh therapeutic approaches to handle medication resistance. Large-scale epidemiological investigations, enhanced molecular surveillance, and the development of integrated parasite control strategies to lower the effect of fasciolosis on cattle output are top priorities in next study.

Supplementary Materials: Not applicable.

Author Contributions: Conceptualization, HKE and MIS; methodology, HKE and MIS; software, HKE and MIS; validation, HKE and MIS; formal analysis, HKE and MIS; resources, HKE and MIS; data curation, HKE and MIS.; writing—original draft preparation,

HKE and MIS; writing—review and editing, HKE and MIS; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data relevant to this manuscript is presented in this manuscript.

Acknowledgments: Authors are thankful to Livestock Experimental Station, Fazilpur for providing support in the execution and completion of the research.

Conflicts of Interest: The authors declare no conflict of interest.

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