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ELISA ASSAYS VERSUS COPROLOGICAL DETECTION OF *FASCIOLA HEPATICA* IN HORSES IN SULAIMANI PROVINCE-NORTH IRAQ

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ABSTRACT

Introduction: Fasciolosis represents a significant economic impact on livestock production in different countries. The gold standard method for diagnosing fasciolosis is currently a coprological examination, based on egg detection in stool. However, this approach is undoubtedly ineffective, especially during the acute phase of the disease. In order to detect all stages of infection, an alternative serological method is used to confirm the infected animals with fasciolosis. Thus, we aimed to identify the pre-patent and patent periods of equine fasciolosis using both copro-microscopical and the ELISA technique in different areas in Garmian region and Sulaimani provinces. **Material and Methods:** A coprological and the sensitive ELISA techniques were used to detect the presence of *Fasciola hepatica* in the infected horses through detection of the serum antibodies against secretary and the excretory antigens from *F. hepatica*. **Results:** The copro-prevalence and seroprevalence of equine fasciolosis were found to be 16% and 58%, respectively, in a total of 50 fecal and 50 blood samples from equine of various ages. **Conclusion:** It was concluded that a serological test had an accurate result for the detection of *Fasciola* infection in horses than the coprological methods; particularly in horses had advanced into the patent period of the infection.

Keywords: Equine; Fasciolosis; ELISA; Prevalence.

INTRODUCTION

Fasciolosis is the major parasitic problem in farm animals that impose economic impact on the livestock production ((Yusuf *et al.*, 2016, Tulu *et al.*, 2018, Zewde *et al.*, 2019). Cosmopolitan fasciolosis is brought on by trematodes of *Fasciola spp*., also known as the liver fluke, which are common in grazing animals and could be found in temperate and high-altitude tropical regions (Borgsteede, 2011, Bennema *et al.*, 2011, Arias *et al.*, 2012a, Beesley *et al.*, 2018, Munita *et al.*, 2019). Wild and domestic ruminants alike are affected by *Fasciola hepatica*, which has an indirect life cycle (Beesley *et al.*, 2018). It also infects monogastric species such as human (Mas-Coma *et al.*, 2018) and horses through ingesting of contaminated food by a larval stage of the fluke, known as metacercaria (Marcos *et al.*, 2007, Pantelouris, 2013,

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Munita *et al.,* 2019). Cercaria is released from snail, the intermediate host of the fluke (*Gulba truncatula*) and then it is transformed into metacercaria (Mas-Coma *et al.,* 2001, Rojo-Vazquez *et al.,* 2012, Moazeni, and Ahmadi, 2016, Neges & Sahle 2018). Climate and other environmental factors, such as the presence of water bodies, wetlands, and pastures, have a significant impact on the distribution of *F. hepatica*. These elements contribute to the development and transmission of free-living fluke stages by creating an environment that is conducive to the intermediate host's growth and reproduction (Charlier *et al.,* 2011, Relf *et al.,* 2011, Olsen *et al.,* 2015), other relevant factors, such as age, breed, stocking rate, and type of farming system, in addition to climate and environmental factors, are also influencing the likelihood of infection (Fox *et al.,* 2011, Kuerpick *et al.,* 2013, Petros *et al.,* 2013, Caminade *et al.,* 2015, Olsen *et al.,* 2015)

Fasciolosis is prevalent in Iraq, and annually causes critical economical losses, in a recent study it was found that the occurrence of fasciolosis in cattle that had been slaughtered was 2.47% in the Erbil province (Ismael and Chalabi, 2021), and 2.30% in the Koya Abattoir that belonging to the Erbil province (Hassan, 2018). Currently, an indirect ELISA technique was used on the serum and milk samples in order to find out how common infections are in small ruminants in the Duhok, Kurdistan Region, Iraq (Mikaeel, 2020). In the other regions of Iraq, such as Basrah, the prevalence of fasciolosis in buffaloes, cattle, sheep, and goats was noted to be 4.8%, 3.3%, 0.72%, and 0.13%, respectively (Mahdi and Al-Baldawi, 1987), and twenty years later, it was confirmed that fasciolosis is still present in Basrah, and the percentage of infection was 2.75% in sheep, 4.42% in cattle, and 16.2% in buffalo (Abdul Wadood, 2005). Interestingly, in a study on 268 donkeys in Bagdad, it was found that the percentage of infection was 4.10%, and 42 *Fasciola hepatica* worms were collected from the livers of slaughtered donkeys (Atia, 2008). Horses were possibly considered a resistant animal to fasciolosis; however, the infected horses showed signs of illness such as low performance, fatigue, diarrhea, decreased appetite, and possibly jaundice (Howell *et al.,* 2020). Diagnosis and detection of fasciolosis are based on clinical signs, copro-microscopical, and immuno-enzymatic tests (Arias *et al.,* 2012b, Rojo-Vazquez *et al.,* 2012, Sanchis *et al.,* 2015). Although many studies in the past investigated F. hepatica in horses for a possible diagnosis, they relied on faecal egg detection (Nansen *et al.,* 1975, Owen, 1977, Apt *et al.,* 1993, Soykan and Öge, 2012). However, because most flukes are unable to reach the mature stage in horses, egg shedding methods are not considered a reliable test for the diagnosis of fasciolosis. As a result, an alternative ELISA test must be considered for identification of the early humoral immune response against the *Fasciola hepatica* (Nansen *et al.,* 1975, Arias *et al.,* 2012a, Howell *et al.,* 2020). Recently, fasciolosis has been identified as a serious risk to public health in a number of endemic nations, including Iraq, where anecdotal reports suggested since 2001, there has been a rise in the number of suspected clinical cases of equine fasciolosis (Atia, 2008). For this reason, this study aimed to determine the pre-patent and patent periods of equine fasciolosis using copro-microscopical and ELISA techniques in different areas in both Garmian region and Sulaimani provinces.

MATERIALS AND METHODS

1. Area of study

The research was confined to 10 different areas (Saida, Rzgary, Hamawmin awa, Kalar, Shehk Langer, Yakhy Maly, Chwartaq, Shekhshaways, Saraw, and Tanjaro), in the Sulaimani province and Garmian region of Kurdistan Region, Iraq. The research territory was situated between 34 and 35 degrees North and 45 and 46 degrees East (Figure 1). This region has four distinct seasons with different rainfall periods in autumn, winter, and early spring that ranged between 827 and 455 mm. These areas are characterized by different habitats, such as the presence of

rivers, meadow pastures, and agricultural lands with high biological diversity.

2. Animals

This study was carried out on 50 horses of different ages from April to July 2021.

3. Collection of fecal samples

The fecal samples were collected from 50 horses, and they were transferred to the clinical pathology laboratory of the Veterinary Education Hospital at College of Veterinary/University of Sulaimani, for coprological examination using the Fecal-Egg-Count-Reduction-Test (FECRT).

4. Blood Samples Collection

The jugular areas of the animals were prepared aseptically for collection of blood samples. Vacutainer tubes were used and approximately (10 mL) of blood was collected from the jugular vein. Then, the samples were centrifuged, the serum isolated and stored at -20 °C prior to use. The stored samples were transported to the laboratory at ambient temperature for the enzyme-linked immunosorbent assay (ELISA).

5. Fecal-Egg-Count-Reduction-Test (FECRT)

The number of fluke eggs per gram of feces was calculated using Eslami's sedimentation method (Eslami *et al.,* 2009). After thoroughly blending the sample, 10 g of feces were weighed out and combined with 500 ml of water in a beaker with the same capacity. The smallest aperture was at the bottom and the largest at the top of three sieves (38 m, 150 m, and 500 m). Slowly passing the water and mixed feces through the sieves was followed by thorough washing with water until the water came out of the bottom sieve clear. The remaining two sieves were washed through the 500-m sieve before it was taken out. The remaining material was backwashed into a 500ml beaker after the 150m sieve was removed, the retentate on the 38m sieve's surface was cleaned, and the 150m sieve was removed. Water was added to the beaker, which was then left to stand for four minutes. After discarding the supernatant and collecting the remaining sediment—roughly 100 ml—the beaker was filled with water and allowed to stand for an additional 4 minutes. Repeating this procedure allowed the supernatant to clear up. The remaining mixture was transferred into a sizable square Petri dish after the supernatant had been poured off to a volume of 100 mL or less without losing any sediment. After 4 drops of Methylene blue were added, the eggs of *F. hepatica* were counted under a dissecting microscope. On the basis of egg morphology and measurements, *Fasciola spp.* eggs were identified, according to (Taira *et al.,* 2003, Bowman, 2014, Taylor *et al.,* 2016, Hendrix and Robinson, 2016, Hardi *et al.,* 2016), (Figure 2). Results were expressed as eggs per gram of feces, as determined by the following formula:

Eggs per gram of feces = Total number of eggs/10

6. Preparation of excretory and secretory antigens for, ELISA: Enzyme-Linked Immunosorbent Assay

The ELISA test was carried out in accordance with Howell *et al.,* 2020. An Immunlon 2HB ELISA plate was coated with 0.5 g/ml of excretory and secretory antigens in 100 l/well (SIGMA-ALDRICH) at pH 9.6 in a carbonate buffer solution of 0.1 M. The plate was sealed with an adhesive plate sealer and incubated at room temperature for one hour before being overnight chilled. Following overnight incubation, the plate samples and reagents were all removed from the fridge and the plate washed. To wash the plate, the contents were flicked out and the wells were filled with wash buffer. This was repeated three times, and after the third fill, the entire procedure—two quick washes and one thorough wash—was repeated after the plate had soaked for five minutes. To remove any remaining liquid, the plate was tapped against absorbent material. 200 ml of blocking buffer (PBS + 2% Tween 20 + 2% BSA) was subsequently added to each well, the adhesive plate sealer was once more used, and the plate was incubated at 37 ^{o}C for an additional hour before being washed as previously mentioned. All serum samples and the

positive and negative serum controls were diluted 1:200 in blocking buffer (containing 2% Marvel), and 100 l of diluted serum was added to each well. 100 ml of blocking buffer (containing 2% Marvel) was added to the conjugate and substrate control wells. The adhesive plate sealer was applied to the plate, which was then incubated at 37 ^{o}C for one hour before being washed as previously mentioned. The conjugate (Goat Anti-Equine IgGT (AbD Serotec)) was diluted 1:20,000 in blocking buffer (containing 2% Marvel), and 100 l was added to each well; 100 l of only blocking buffer was added to the substrate control wells. Reapplying the adhesive plate sealer, the plate was incubated at 37 ^{o}C for an additional hour, and then washed as before. Each well received 100 l of TMB (tetramethylbenzidine), and the plate was incubated for 20 minutes in the dark. Following the addition of 100 l of 0.5 M HCl to each well to stop the reaction, the color change was detected at 450 nm using an automatic ELISA reader (TECAN, INFINATE F50). The optical density (OD), which is used to represent the results, is calculated using repeat samples and expressed as a percentage of the positive control using the following formula:

$$\text{Percent positive} = \frac{\text{mean OD of test serum}}{\text{mean OD of +control}}$$

On each plate, both positive and negative serum controls, conjugate controls, and substrate controls were used, and these were all tested in triplicate. Each test serum sample was tested at least in duplicate on each ELISA test, and each plate was repeated three times.

7. Statistical analysis

For statistical analysis, GraphPad Prism (version 5) software was employed. A P value of 0.05 was used as the threshold for statistical significance in an unpaired T test.

RESULTS

In the current study, we created and assessed an ELISA for the detection of Fasciola hepatica in horses from various locations in the province of Sulaimani and Garmian region. According to their ages, the animals were divided into three groups: young (1-3 years old), adult (3-5 years old), and the aged group (over 5 years old). The prevalence of liver fluke infection varied for each technique used for the detection of fasciolosis. In the coprological test, only 8 samples were positive (16%), in which different numbers of parasitic eggs were detected in each group. In the ELISA technique, it was found that a larger number of horses had a positive antibody in their serum. The number of infected horses was 29; this raised the prevalence of equine fasciolosis to 58%. Using the ELISA test, it indicated the pre-patent infection of fasciolosis in contrast to the coprological examination, which could not detect the pre-patent period of infection. Moreover, the correlation between ages and the infection could be presented, in which horses older than 2 years showed positive immune responses to fasciolosis, in contrast to the coprological tests, which showed negative results for almost most of the samples (Tables 1, 2, and 3). These findings indicate the prevalence of fasciolosis in different areas of northern Iraq. This was confirming that horses in different areas of this region were exposed to fasciolosis, and became a carrier vehicle for this parasite, particularly young horses, whose ages ranged from 1 to 5 years old. However, there was a pre-patent period of infection in horses older than 5 years, but no eggs were found in their faeces. This finding indicated that the aged horses had more resistance to fasciolosis than the young, and this possibly referred to the previous infection with liver fluke. Previous infection with liver fluke could result in the production of specific antibodies that remain for several months, and subsequently protect the horse from recurrent infection (Figure 3).

Both techniques showed positive cases of fasciolosis in equine species with different accuracy. In the ELISA technique, positive antigens were found in all age groups. Interestingly, all the individual ages, from very young (2 years old) to aged horses (12 years old), showed positive results for the antigen of *Fasciola hepatica*. This finding

suggests that there is no age preference for equine fasciolosis and that all ages are susceptible to infection. Although different horses produced different results, some of them, aged 2 to 3.5 years old, produced positive results in both techniques. While a larger number of horses of almost all ages showed positive results for the ELISA test (Figure 4).

Finally, it was found that the ELISA technique showed an accurate method for detection of equine fasciolosis in both the pre-patient and the post-patient periods of all the exposed horses to *Fasciola hepatica*. This finding of greater sensitivity could postulate the idea of replacing the coprological test by an ELISA for detection of liver fluke infection. The accuracy of ELISA was up to 100%, and it was statistically significant (using an unpaired T test) than the coprological assay, as they were up to four-fold more accurate (58%) than the coprological test (16%) (Figure 5).

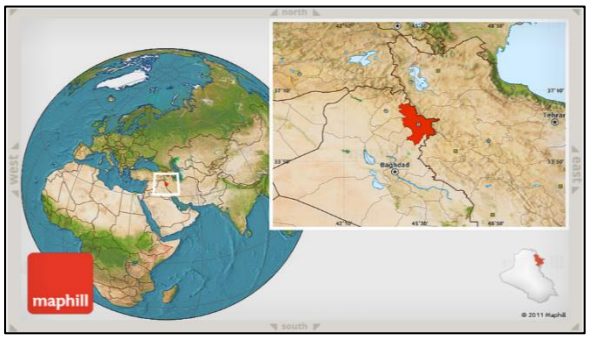

Figure 1: Shows the geographical location of both Sulaimani and the Garmian provinces on the map (red patch), where faecal and blood samples were collected from horses for coprological and ELISA tests. http://www.maphill.com/ iraq/ sulaymaniyah/location-maps/satellite-map/)

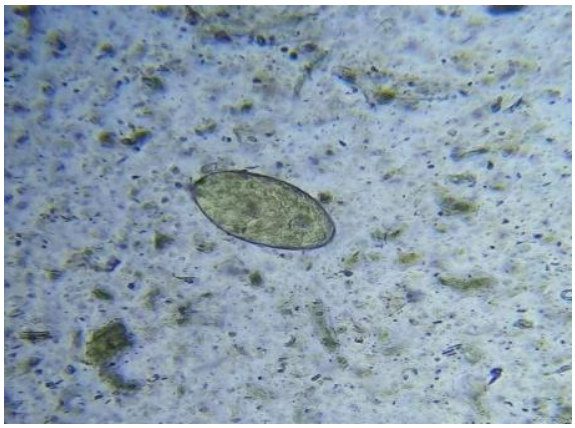

Figure 2: Egg of *Fasciola Hepatica*, identified during coprological examination (40x magnification)

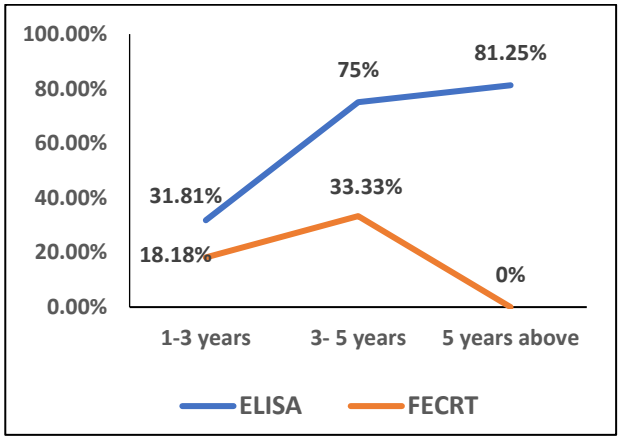

Figure 3: Shows the age-related percentages of the positive cases in all the three groups using both techniques, ELISA and FECRT for detection fasciolosis in equine.

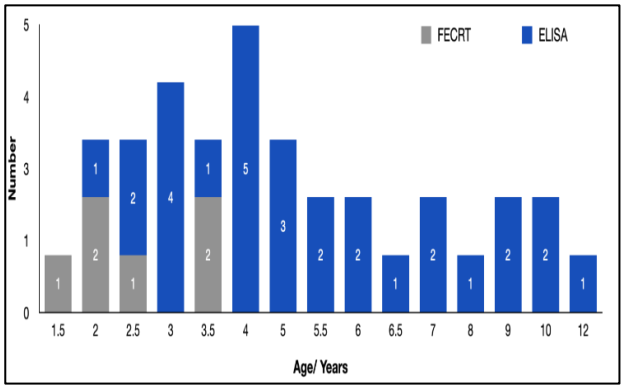

Figure 4: Illustrates the relation between the ages and the positive results for both coprological and ELISA tests.

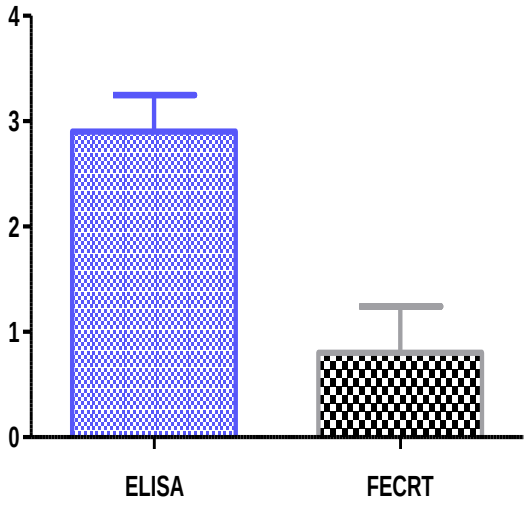

Figure 5: Shows the accuracy of both tests for detecting the parasitic infestation. There was a significant difference between these two tests when using un-paired T test, (P value < 0.05), (Error bars = SD), Graphpad prism.

Table 1: Results of FECRT Assay of Different Ages

Ages	# of samples	# of positive	Percentage
1-3 years	22	4	18.18%
3-5 years	12	4	33.33%
Above 5 years	16	0	0

Table 2: The Results of ELISA Assay of Different Ages

Ages	# of samples	# of positive	Percentage
1-3 years	22	7	31.81%
3-5 years	12	9	75%
Above 5 years	16	13	81.25%

Table 3: Shows number of faecal samples taken from horses in ten different places with information about number of eggs, sex, age, and the result of positivity and negativity by FECRT. (M) is the male and (F) is the female horses. (Y) is the year of age.

Number	Number of eggs	Sex	Age	Area	Results
1.	0	M	3 Y	Rzgary	-ve
2.	0	M	3 Y	Rzgary	-ve
3.	0	M	12 Y	Rzgary	-ve
4.	0	M	2 Y	Rzgary	-ve
5.	0	M	2.5 Y	Rzgary	-ve
6.	0	F	3.5 Y	Saida	-ve
7.	0	M	4 Y	Saida	-ve
8.	0	M	4 Y	Saida	-ve
9.	0	F	2.5 Y	Hamawminawa	-ve
10.	0	M	10 Y	Hamawminawa	-ve
11.	0	M	3 Y	Hamawminawa	-ve
12.	0	M	2.5 Y	Hamawminawa	-ve
13.	0	F	9 Y	Shex langer	-ve
14.	0	M	9 Y	Shex langer	-ve
15.	0	M	4 Y	Shex langer	-ve
16.	0	F	12 Y	Shex langer	-ve
17.	0	F	10 Y	Kalar	-ve
18.	0	M	11 Y	Kalar	-ve
19.	0	M	1 Y	Kalar	-ve
20.	0	F	6.5 Y	Kalar	-ve
21.	0	F	7 Y	Kalar	-ve
22.	0	F	4 Y	Shexshaways	-ve
23.	0	F	2 Y	Shexshaways	-ve
24.	0	M	7 Y	Shexshaways	-ve
25.	0	M	5 Y	Shexshaways	-ve
26.	0	M	2.5 Y	Shexshaways	-ve
27.	0	F	6 Y	Yaxy maly	-ve
28.	200	M	3.5 Y	Yaxy maly	+ve
29.	200	M	2 Y	Yaxy maly	+ve
30.	150	F	4 Y	Yaxy maly	+ve
31.	0	M	5 Y	Yaxy maly	-ve
32.	0	M	3 Y	Yaxy maly	-ve
33.	0	M	5 Y	Yaxy maly	-ve
34.	0	M	7 Y	Saraw	-ve
35.	0	F	3 Y	Saraw	-ve
36.	0	F	2 Y	Saraw	-ve
37.	224	M	4 Y	Saraw	+ve
38.	166	M	3.5 Y	Saraw	+ve
39.	78	F	2 Y	Saraw	+ve
40.	0	F	8 Y	Tanjaro	-ve
41.	0	F	3 Y	Tanjaro	-ve
42.	0	M	8 Y	Tanjaro	-ve
43.	216	M	1.5 Y	Tanjaro	+ve
44.	371	M	2.5 Y	Tanjaro	+ve
45.	0	M	4 Y	Chwartaq	-ve
46.	0	F	5 Y	Chwartaq	-ve
47.	0	F	3 Y	Chwartaq	-ve
48.	0	M	6 Y	Chwartaq	-ve
49.	0	M	7 Y	Chwartaq	-ve
50.	0	F	3 Y	Chwartaq	-ve

DISCUSSION

Numerous endemic countries face a serious threat to public health from fasciolosis, with millions of people either at risk of infection or already infected (Tolan, 2011, Hardi *et al.*, 2016). According to the current study, fasciolosis was more common using FCRCT techniques (16% vs. 58% using ELISA). Similarly, this was reported by different studies with different results, such as in a study in Egypt, where it was 1.5% (Haridy *et al.*, 2002). In Van, Turkey, it was 5.8% (Karaca *et al.*, 2005). In Konya, Turkey, it was 3.6% (Uslu and Guçlu, 2007), in Turkey's

Black Sea region, it was 4.82% (Umur and Acici, 2009). In Turkey, it was 8.5% (Acici *et al.*, 2013), in Malaysia it was 7.50% (Zainalabidin *et al.*, 2015), in Ireland, it was 3% (Quigley *et al.*, 2017), in Chile, it was 8.06% (Muñoz *et al.*, 2020) and in Ethiopia, it was 13.7% (Mathewos *et al.*, 2021). Meanwhile, higher incidences were recorded by other researchers such as in a study in Iran, it reached up to 50% (Eslami *et al.*, 2009), and in Ethiopia, it was 44.4% (Getachew *et al.*, 2010).

In addition to the coprological test, an ELISA test has been created to detect serum antibodies against secretory and excretory enzymes from *F. hepatica* in order to determine the presence of the pathogen in horses (Walsh *et al.*, 2021). Thus, using ELISA conferred advantages over the coprological technique because it could detect the parasitic infection in the pre-patent period and because the circulating antibodies could stay in the blood for several months after successful deworming, it would enable early detection of fasciolosis (Nansen *et al.*, 1975, Owen, 1977, Salimi-Bejestani *et al.*, 2005, and Arias *et al.*, 2012a). Meanwhile, the coprological technique is less accurate for detecting fasciolosis due to the absence of eggs in the faeces, particularly during the migration stage, when immature flukes do not produce eggs, though the eggs could occasionally be observed during mature stages due to their irregular shape (Dorchies, 2007, Mursyidah *et al.*, 2017). In this study, it was found that the accuracy of ELISA showed a higher incidence of fasciolosis than the coprological examination. In a similar study in Turkey, according to reports, serological approaches had a higher prevalence of fasciolosis than the coprological approach (Yildirim *et al.*, 2007, Pinilla *et al.*, 2020). The cause of this discrepancy, in contrast to the coprological test, can detect parasite eggs after 12 weeks, circulating antibodies against *Fasciola* species can be detected much earlier, and the circulating antibody titer in infected cattle can last for up to six months after infestation (Castro *et al.*, 2000, Mohammed *et al.*, 2018).

In the current study, using the ELISA test indicated a higher incidence of infection (58%), and this higher incidence is also recorded by other studies. In a study, in post-mortem findings in Uruguay, it was found that the percentage of liver infestation in equines was 59% (Sanchis *et al.*, 2015). As well as in other studies in Turkey, it was 5.1% (Soykan and Öge 2012), In Holeta, Ethiopia, it was 24% (Ayana *et al.*, 2017), in Jigjiga, Ethiopia, it was 4.5% (Abdulahi *et al.*, 2017), in and around Mekelle, Ethiopia, it was 1.6% (Gebreyohans *et al.*, 2017), in Tenta Woreda-Ethiopia it was 2.2% (Getahun and Kassa, 2017), in Konya-Turkey it was 6.2% (Uslu and Guçlu, 2007), in Gondar Town-Ethiopia it was 5.7% (Mezgebu *et al.*, 2013). The temperate climate typical of Ireland can be used to explain the relatively high prevalence reported in the current study, as it creates ideal conditions for G. truncatula and the environmental stages of F. hepatica to flourish and infect animals (Munita *et al.*, 2019). The smaller sample sizes in earlier studies, which raise the margin of error in the studies, are primarily to blame for the higher prevalence in those studies (Naing *et al.*, 2006, Hardi *et al.*, 2019). However, the results were lower than those obtained by (Simsek *et al.*, 2007 and Yildirim *et al.*, 2007, and Ali *et al.*, 2021), which were 60.5%, 65.2% ,and 49.48%, in Elazig and Kayseri, Turkey, Sharazur District Kurdistan- Iraq, respectively. This study determined that Garmian region was free from horse fasciolosis, and all positive samples were found in Sharazur district. This may be due to horses in the Garmian region being raised indoors and fed concentrated feed and not allowed to graze on pasture because most people raise horses for entertainment and could be primarily due to variations in climatic and ecological conditions like altitude, rainfall, and temperature (Yusuf *et al.*, 2016, Hardi *et al.*, 2019). Fasciola spp. prevalence has been reported to change over time, primarily due to changes in rainfall amount and pattern (Ali *et al.*, 2021). However, Sharazur has rather more rain

fall and horses raised outdoors, which make conditions more suitable than Garmian (Hardi *et al.*, 2019). These may be the crucial points to focus on in future works. The rate of disease propagation is significantly affected by age. It's important to note that older animals had a higher prevalence of fasciolosis (older than 3 years). These findings are in line with earlier findings and point to a lifelong effect of infection on horses (Arias *et al.*, 2012b, Acici *et al.*, 2013, Getachew *et al.*, 2010 and Rita *et al.*, 2017). Farmers' feeding practices and the fact that animals older than five years were allowed to graze freely on the farm, increasing their exposure to the infestation's causative agent, are likely to account for the differences (Ahmad-Najib *et al.*, 2021). A larger-scale test that can be used to estimate the prevalence of F. hepatica in various horse populations will be provided by further research and development of this ELISA test, which will be helpful for both diagnosing F. hepatica infection in specific horses and providing that test.

CONCLUSION

It was determined that horses grazing on ruminant grazing pastures in northern Iraq should be monitored for liver fluke exposure. Using the serological test has a more accurate result for detection of fasciolosis infection in horses compared to the chemical sedimentation method. Regular and strategic deworming programs with effective anthelmintics should be carried out on a regular basis to reduce the risk of epidemiological and economic risk factors.

CONFLICT OF INTEREST

The authors have not disclosed any conflicts of interest.

ACKNOWLEDGEMENT

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ETHICAL APPROVAL

The procedure performed in this study was approved by the College of Veterinary Medicine scientific research committee, the University of Sulaimani, and the Kurdistan Regional Government of Kurdistan and Iraq.

STATEMENT OF HUMAN AND ANIMAL RIGHTS

All study procedures and approaches were carried out in accordance with the ethics principles approved by the College of Veterinary Medicine Research Committee, the University of Sulaimani, the Kurdistan Regional Government, and Iraq.

FINANCIAL DISCLOSURE STATEMENT

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