OPEN ACCESS
Advances in Animal and Veterinary Sciences
Research Article

# Molecular Sequencing Analysis of *Fasciola* Spp. in Sheep

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**Abstract** | This study aims to investigate the prevalence rate of *Fasciola* in livers of 361 slaughtered sheep at Basra province (Iraq) with molecular testing and phylogenetic analysis of obtained flukes in the National Center For Biotechnology Information (NCBI). The findings showed that 8.03% of livers were infected with *Fasciola* spp., in which, juvenile flukes were observed significantly (79.31%) when compared to adults (20.69%). Based on morphology, all adult flukes were diagnosed as *Fasciola.* Accordingly, positive rate, risk and Odd ratio of fasciolosis were reported significantly in sheep of larger than 3 years than those of larger than 2-3 years, 1-2 years and sheep of less than 1 year. Concerning sex, no variation was detected between female (9.02%) and male (5.26%) sheep; however, females were appeared at higher risk of infection males. Molecular examination using the PCR assay demonstrated that 93.1% of samples were *Fasciola*; whereas, phylogenetic analysis of five isolates indicated its identity to Saudi Arabian (MN559388.1) *F. hepatica* isolate. In conclusion, Molecular and phylogenetic analysis of ovine liver flukes indicated worthily that *F. hepatica* was the more prevalent *Fasciola* species in sheep of Basra province. In addition, our results provided an initial basic data for monitoring this potentially important parasite in field. However, the main limitations of the present study include the low number of examined animals, short period of study, and disapproving of some owners to examine their slaughtered animals. Therefore, it is necessary to develop the suitable parasite control measures (e.g. controlling the snails, judicious annual using of flukecide, frequent examination of fecal samples of field animals, and destroying of infected livers) and evaluation the local epidemiology of fasciolosis. Also, wider surveys including different areas and animals with using the advanced diagnostic assay in combination with morphology should be conducted.

**Keywords** | Fasciolosis, Ovine liver fluke, Conventional PCR, Phylogeny, *Fasciola hepatica*, Iraq

**Received** | June 14, 2024; **Accepted** | July 26, 2024; **Published** | August 22, 2024
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**Citation** | Essa IM, Azzal GY, Abdulwahid AT (2024). Molecular sequencing analysis of *fasciola* spp. in sheep. Adv. Anim. Vet. Sci. 12(10): 1846-1852.
**DOI** | https://dx.doi.org/10.17582/journal.aavs/2024/12.10.1846.1852
**ISSN (Online)** | 2307-8316; **ISSN (Print)** | 2309-3331



## INTRODUCTION

*Fasciola*, commonly known as liver fluke, is a parasitic flatworm which classify under the Plagiorchiida Order of the Trematoda Class in the Platyhelminthes Phylum. The genus of *Fasciola* includes mainly *F. hepatica* Linnaeus, 1758, and *F. gigantica* Cobbold, 1855 in addition to a number of invalid species such as *F. indica*, *F. californica*, *F. halli* and *F. nyanzae* (Itagaki *et al*., 2022; Madsen *et al*., 2022). Wide range of animal species and humans can infect with *Fasciola* resulting in a public health concern and severe economic losses in livestock industry (Beesley *et al*., 2018; Regasa *et al*., 2021; Zerna *et al*., 2021). Lifecycle of *Fasciola* comprises five phases; egg (pass through feces of mammalian host), miracidia (free-swimming and infect the intermediate host), cercariae (erupt from snail host and attached

to aquatic plants), metacercarial cysts (in aquatic plants) and adult fluke (mammalian host). After ingestion, young flukes were excyst in duodenum, penetrate intestinal wall, enter the peritoneal cavity, and migrate to liver penetrating the capsule and tunnel of parenchyma. After growing and destroying tissues, flukes enter the bile ducts and occasionally the gallbladder where they mature and begin produce eggs (Phalee *et al*., 2015; Moazeni *et al*., 2016; Lalor *et al*., 2021; Stuen *et al*., 2022).

According to amount of ingested metacercariae, clinical symptoms might range from mild to severe devastating illness. Acute form of infection is manifested by sudden death, anemia, and painful-distended abdomen could occur seasonally in sheep. While the chronic form of disease might occur at any season particularly winter causing a submandibular edema, unthriftiness, anemia and decreased milk yield (Constable *et al*., 2016; Forbes, 2017). Based on stage of disease, diagnosis can be done traditionally using the fecal sedimentation test especially in chronic and subacute infections (Kajugu *et al*., 2015). However, serological enzyme-linked immunosorbent assays (ELISAs) and molecular techniques such as polymerase chain reaction (PCR) have provided reliable tools in last decades (Webb *et al*., 2018; Caravedo *et al*., 2020). Furthermore, genetic research has determined the origin, toxicity, evolution, development of parasite and resistance to various anthelmintics (Cwiklinski *et al*., 2015; Beesley *et al*., 2017).

In Iraq, molecular prevalence of *Fasciola* have been studied in Wasit (Abdulwahed *et al*., 2019), Mosul (Hamoo *et al*., 2019), Sulaymaniyah (Raoof *et al*., 2020), and Erbil (Muhammad and Hassan, 2021) provinces. Therefore, this study aims to investigate the prevalence rate of *Fasciola* in livers of 361 slaughtered sheep at Basra province (Iraq) with molecular testing and phylogenetic analysis of obtained flukes in NCBI.

**Table 1:** Total results for gross examination of livers of 361 sheep.

| Animal | Total No. | Positives [No. (%)] | | |
|---|---|---|---|---|
| | | Total | Juvenile worms | Adult worms |
| Sheep | 361 | 29 (8.03%) | 23 (79.31%) * | 6 (20.69%) |
| p-value | | | 0.0263 S | |

Significance (S) *; Non-significance (NS)

## MATERIALS AND METHODS

### Samples

During November (2023) to February (2024), livers of totally 361 slaughtered sheep at the official and private abattoirs in Basra province (Iraq) were examined grossly to detect the presence of flukes that collected initially in plastic petridishes contained phosphate buffered saline (PBS).

### Morphology

At laboratory, the collected flukes were examined directly based on the external characteristics as described by Rokni *et al*. (2010).

### Molecular Assay

Firstly, the samples of liver fluke were thawed in water path, washed extensively in PBS, and subjected to steps of the DNAs extraction as described in the Protocol B of G-Spin™ Total DNA Extraction Kit (Intron Biotechnology, Korea). After estimation of purity and concentration of extracted DNAs using the Nanodrop System (Thermo-Scientific, UK), the MasterMix tubes were prepared following the manufacturer instructions of the AccuPower™ PCR PreMix (Bioneer, Korea) using one set of designed primers: ISR-F (5´-ATC ACT GAT GGG GTG CCT AC-3´) and ISR-R (5´-CCG GAT ACA TTA GGG AAA CG-3´) by targeting the *18S rRNA* gene of *Fasciola* spp. in the NCBI-GenBank (MW620063.1) isolate at a final volume of 20 µl. Then, the MasterMix tubes were amplified in the Thermal Cycler (Bio-Rad, USA) at the following conditions: 1 cycle for initial denaturation (95˚C / 5 minutes), 35 cycles for denaturation (95˚C / 30 seconds), annealing (56˚C / 30 seconds) and extension (72˚C / 1 minute), and 1 cycle for final extension (72˚C / 7 minutes). Electrophoresis of PCR products were performed in agarose-gel (1.5%) stained with Ethidium Bromide at 100 Volt and 80 Am for 1 hour. According to size of ladder marker, positive samples were identified under UV transilluminator at ≈592 bp.

For phylogeny, DNAs of five positive local *Fasciola* isolates were sequenced by the Macrogen Company (Korea), and the data were submitted in the NCBI-GenBank, and analysed using the MEGA Software (*version x*) to detect the percentage of identity between the local study isolates and global NCBI-BLAST isolates.

### Statistical Analysis

The *t*-test, Odd ratio and risk were served in the GraphPad Prism Software (*version 6.0.1*) to detect significant differences at a level of P<0.05 (*), P<0.01 (**), P<0.001 (***) and P<0.0001 (****), (Gharban, 2023).

## RESULTS AND DISCUSSION

In the present study, 8.03% of sheep were infected with liver flukes with significant prevalence (P<0.0263) of juvenile flukes (79.31%) when compared to adults (20.69%). Based on morphology, all adult flukes were diagnosed as *Fasciola* (Table 1). In comparison to national studies, there was 0.28% in Kirkuk (Abass *et al*., 2018), 55.71% in Sulaimani (Ali *et al*., 2021), 1.76% in Duhok (Nerway *et al*., 2021), 5.5% in Salah al-Din (Sulieman *et al*., 2021), 10% in Mosul (Al-Lahaibi *et al*., 2023), and 2% in Erbil (Koyee *et al*., 2023); whereas globally, there was 13.2% in Ethiopia

(Jittapalapong, 2007), 3.09% in India (Shende *et al*., 2007), 6.6% in Iran (Khanjari *et al*., 2014), 14.7% in Egypt (Amer *et al*., 2016), 100% in Indonesia (Kusumarini *et al*., 2020) and 26.2% in Turkey (Ayvazoğlu *et al*., 2023). This variation might attribute to number of tested samples, topographical properties, management system, climatic conditions, degree of exposure to intermediate host, and therapeutic / prevention schemes applied for controlling infection.

**Table 2:** Distribution of liver flukes according to animal risk factor (age and sex).

| Factor | Category | Total No. | Positive | | Risk | Odds Ratio |
|---|---|---|---|---|---|---|
| | | | No. | % | | |
| Age (Year) | < 1 | 117 | 0 | 0 | 0 | 0 |
| | 1-2 | 148 | 4 | 2.7 | 0.1 | 0.079 |
| | >2-3 | 62 | 14 | 22.58 | 2.756 | 3.252 |
| | >3 | 34 | 11 | 32.35 * | 3.781 **** | 5.096 **** |
| p-value | | | 0.0426 S | | 0.0001 S | 0.0001 S |
| Sex | Female | 266 | 24 | 9.02 | 1.711 **** | 1.781 **** |
| | Male | 95 | 5 | 5.26 | 0.284 | 0.556 |
| p-value | | | 0.0621 NS | | 0.0001 S | 0.0001 S |

Significance (S) *; Non-significance (NS).

According to animal risk factors, values of positive rate, Odd ratio and risk were elevated significantly in sheep of >3 years old (32.35%, 3.781 and 5.096, respectively) in comparison to sheep aged >2-3 years (22.58%, 2.756 and 3.252, respectively), 1-2 years (2.7%, 0.1 and 0.079, respectively) and < 1 year (0%, 0 and 0, respectively), (Table 2). Worldwide, several researchers demonstrated that *Fasciola* infection and the risk of parasite have increased significantly with advancing age of study sheep and being higher at > 3 years (Ahmed *et al*., 2007; Rinaldi *et al*., 2015; Denizhan and BİÇek, 2018). This could explain because that either older animals might expose largely and frequently to source of infection or lowering resistance to infection at advanced ages. In contrast, the findings of another study found the lack of significant differences between the study age groups including <1 (8.33%), 1-2 (6.66%), and >2 (12.5%) years (Al-Lahaibi *et al*., 2023).

Concerning the sex of study sheep, no significant variation (P<0.0621) was seen between female (9.02%) and male (5.26%) sheep; however, females were appeared at higher risk of infection than males. These findings were similar with other researchers (Asadian *et al*., 2013; Denizhan and BİÇek, 2018; Al-Lahaibi *et al*., 2023). In contrast, other researchers found that females have higher rate of fasciolosis than males (Isah, 2019; Muhammad and Hassan, 2021). The increasing risk of fasciolosis in females might be attributed to either study female are older in age or immunity of shhep is attenuated due to stressful conditions of breeding and milk production.

Molecular examination using the conventional PCR assay indicated 93.1% (27 / 29) positive samples to *Fasciola* spp. including 91.3% (21/23) juvenile flukes and 100% (6/6) adult flukes (Table 3, Figure 1). Although, *Fasciola* species can be distinguished based on their morphological criteria, precise differentiation of two flukes is often difficult due to morphological variations among different isolates (Rokni *et al*., 2010; Yakhchali *et al*., 2015). In addition, Marcilla *et al*. (2002) thought that neither parasitological plus clinical tests nor immunological assays can differentiate between *Fasciola* species. Therefore, DNA-based diagnostic techniques can confirm morphometric tools and facilitate the task of species differentiation. This could because genotyping characters are not influenced by ecological and geographical factors. In addition, molecular methods can be used to understand the nature and extent of inter- and intra-specific variations in *Fasciola* species (Qureshi *et al*., 2019; Das *et al*., 2022).

**Table 3:** Total results of molecular examination of 29 suspected samples as *Fasciola* spp. by PCR.

| Animal | Total No. | Positives [No. (%)] | | |
|---|---|---|---|---|
| | | Total | Juvenile worms (Total no. 23) | Adult worms (Total no. 6) |
| Sheep | 29 | 27 (93.1%) | 21 (91.3%) * | 6 (100%) |
| p-value | | | 0.0482 S | |

Significance (S) *; Non-significance (NS).

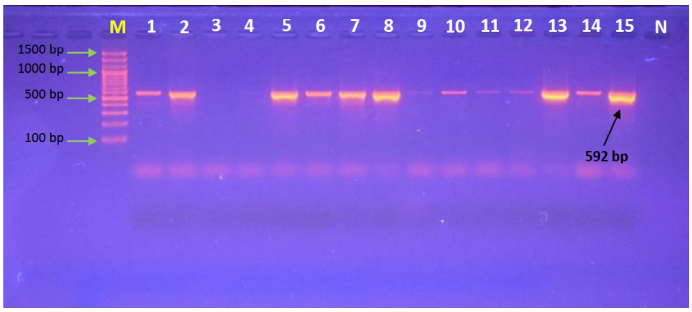


**Figure 1:** Electrophoresis of some PCR products targeting *18S rRNA* gene of *Fasciola*;**Lane (M):** Ladder marker (100-1500 bp)**; Lanes (1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15):** Positive samples; **Lanes (3 and 4):** Negative samples; **Lane (N):** Negative control.

In sheep, different genes have been used to identify *Fasciola* and confirming its species such as *Cox1* (Muhammad and Hassan, 2021; Wang *et al*., 2022), *ITS* (Kobra *et al*., 2018; Alsulami *et al*., 2023), and *18S rRNA* (Saadatnia *et al*., 2022; Sultana *et al*., 2022) genes. Sequencing data of five local *Fasciola* isolates were submitted in the NCBI-GenBank under the names of *Fasciola hepatica* IQ-1 Sheep isolate, *Fasciola hepatica* IQ-2 Sheep isolate, *Fasciola hepatica* IQ-3 Sheep isolate, *Fasciola hepatica* IQ-4 Sheep isolate, and *Fasciola hepatica* IQ-5 Sheep isolate. Phylogenetic tree analysis detected the presence of significant identity between the study *Fasciola* isolates and the NCBI-GenBank

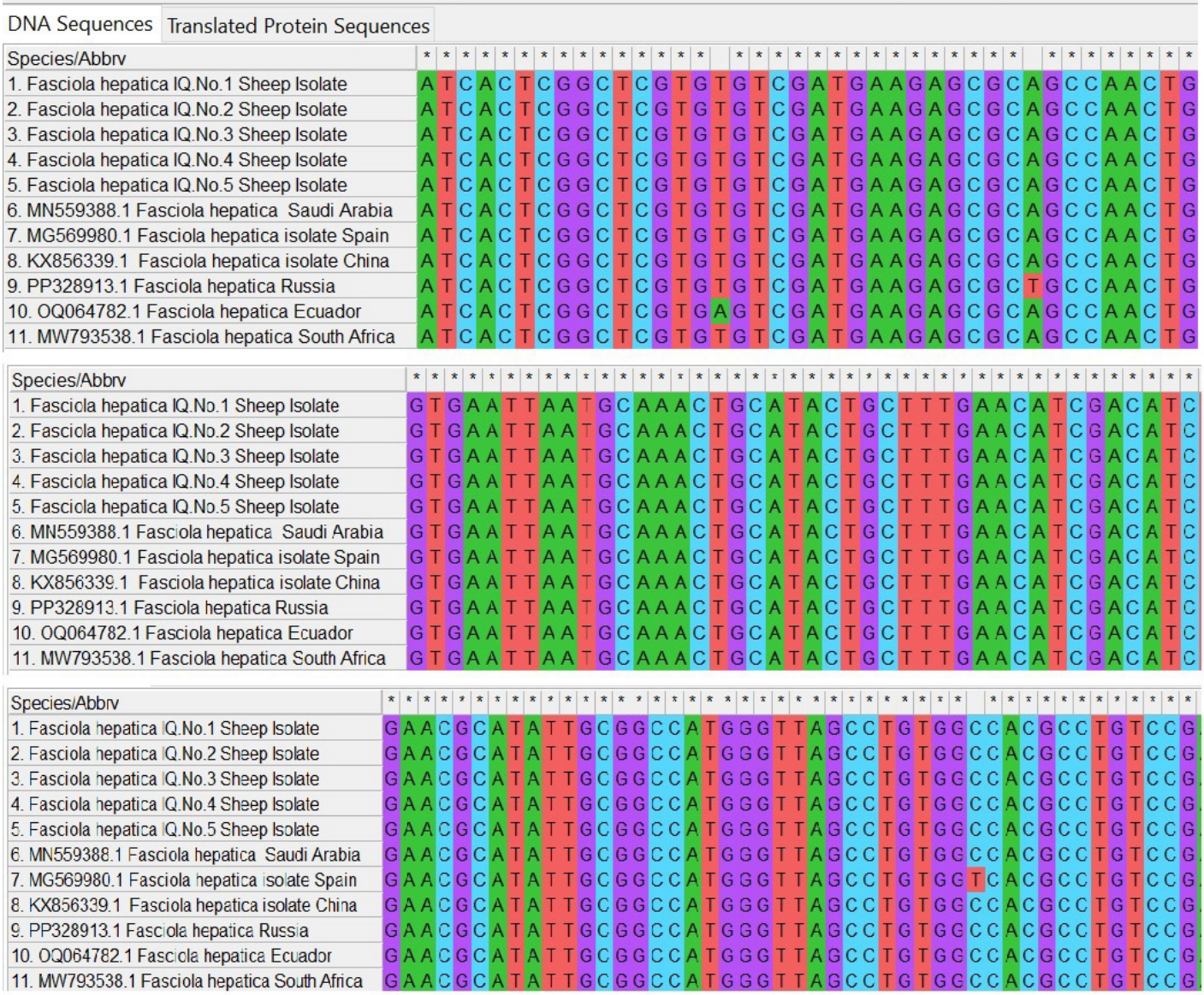


**Figure 2:** Multiple sequence alignment of local and NCBI-GenBank *Fasciola* isolates showing nucleotide similarity and substitution mutations at *18S rRNA* gene.

Saudi Arabian (MN559388.1) *F. hepatica* at 99.72-99.78% (Figure 2) and a homology sequence identity at 99.76-99.78% (Table 4, Figure 3). These results demonstrated that *F. hepatica* is the most prevalent *Fasciola* species in sheep of Basra province as reported in other Iraqi (Koyee *et al.*, 2023; Othman *et al.*, 2023; Gharban *et al.*, 2024) and global (Alsulami *et al.*, 2023; Celik *et al.*, 2023; Gröning *et al.*, 2023) studies.

**Table 4:** Homology sequence identity between the local *Fasciola* isolates and the NCBI-GenBank *Fasciola* isolates.

| Local isolate | | | NCBI-BLAST isolate | | |
|---|---|---|---|---|---|
| No. | Access No. | Band size | Country | Access No. | Identity |
| IQ. No. 1 | PP779808.1 | 511 bp | Saudi Arabia | MN559388.1 | 99.72% |
| IQ. No. 2 | PP779809.1 | 504 bp | Saudi Arabia | MN559388.1 | 99.78% |
| IQ. No. 3 | PP779810.1 | 499 bp | Saudi Arabia | MN559388.1 | 99.72% |
| IQ. No. 4 | PP779811.1 | 514 bp | Saudi Arabia | MN559388.1 | 99.72% |
| IQ. No. 5 | PP779812.1 | 510 bp | Saudi Arabia | MN559388.1 | 99.78% |

## CONCLUSIONS AND RECOMMENDATIONS

Molecular and phylogenetic analysis of ovine liver flukes indicated worthily that *F. hepatica* was the more prevalent *Fasciola* species in sheep of Basra province. Also, our results provided an initial basic data for monitoring this potentially important parasite in field. However, the main limitations of the present study include the low number of examined animals, short period of study, and disapproving of some owners to examine their slaughtered animals. Therefore, it is necessary to develop the suitable parasite control measures (e.g. controlling the snails, judicious annual using of flukecide, frequent examination of fecal samples of field animals, and destroying of infected livers) and evaluation the local epidemiology of fasciolosis. Also, wider surveys including different areas and animals with using the advanced diagnostic assay in combination with morphology should be conducted.

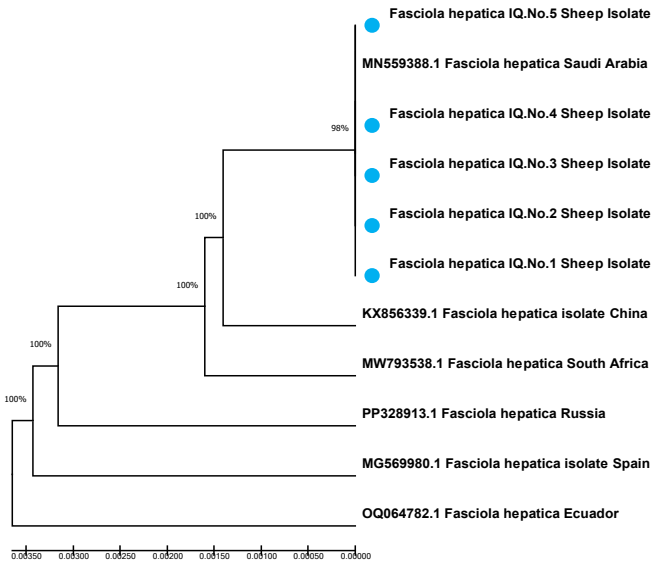


**Figure 3:** Phylogenetic tree analysis of local and NCBI-GenBank *Fasciola* isolates showing the level of identity at 99.72-99.78%.

## ACKNOWLEDGMENTS

Authors thank the Head and Staff of the Department of Parasitology (College of Veterinary Medicine, University of Basrah) and all veterinarians who contributed in collection of liver flukes.

## NOVELTY STATEMENT

The current study provided a new recent data about the molecular prevalence of ovine fasciolosis in Basra province (Iraq), and analysed phylogenetically the local isolates to detect their identity with the global isolate for first time in study areas.

## AUTHOR'S CONTRIBUTIONS

IME: Gross examination of slaughtered sheep, collection of liver flukes, and phylogenetic analysis of local *Fasciola* spp. isolates. GYAA: Morphological examination of collected flukes, and statistical analysis of obtained results. ATA: Molecular analysis of collected flukes.

All authors have approved the final copy of the manuscript.

### Funding

This study didn't receive any funding support

### Ethics Approve and Participant Consent

The current study was licensed by the Scientific Committee of the Department of Parasitology in the College of Veterinary Medicine (University of Basrah, Basra, Iraq).

### Avaialability of Data and Materials

All obtained data were included in this manuscript.

### Conflict of Interest

The authors have declared no conflict of interest.

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