Original Article

Prevalence, Molecular Identification, and Phylogenetic Analysis of *Strongylus equinus* in Horses in Al-Muthanna Province, Iraq

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How to Cite This Article Jasim, H. J., Alkaabawi, N. A. M., Abbass, Z. A. A., & Mohammed alomari. M. M. (2026). Prevalence, Molecular Identification, and Phylogenetic Analysis of *Strongylus equinus* in Horses in Al-Muthanna Province, Iraq. *Iranian Journal of Veterinary Medicine, 20*(2), 311-320. http://dx.doi.org/10.32598/ijvm.20.2.1005901

http://dx.doi.org/10.32598/ijvm.20.2.1005901

ABSTRACT

Background: Gastrointestinal parasites, particularly *Strongylus* spp., represent a critical challenge to equine health and productivity in developing nations.

Objectives: This study aims to provide the first molecular and phylogenetic characterization of *Strongylus equinus* in horses in Iraq, using the internal transcribed spacer (ITS-1) region of rDNA as a marker for species-level identification. It has not been reported previously in the Middle East. Additionally, it establishes baseline data on age, sex, and season-linked risk factors influencing *Strongylus* spp. prevalence in Al-Muthanna Province, Iraq, a previously unstudied region, thereby addressing critical gaps in the epidemiology of equine parasites.

Methods: The flotation technique was performed on 118 horse fecal samples, randomly collected from stables in Al-Muthanna Governorate, Southern Iraq, from January to the end of October 2023. Then, polymerase chain reaction (PCR) was performed using species-specific primers (StrongF/StrongR) for 50 samples. Three high-quality PCR products with strong band intensity from local *S. equinus* isolates were sent for sequencing.

Results: The epidemiological analysis discovered that 50(42.3%) out of 118 horses were positive for *Strongylus* spp. infection via the flotation technique. Infection rates were significantly higher in horses under 4 years of age (57.1%); this difference was not statistically significant ($P>0.05$). Also, the highest prevalence rate was recorded during March (83.3%), and in females (56.2%) ($P\leq0.05$). Moreover, PCR confirmed the presence of *S. equinus* in 22(44%) out of 50 morphologically positive samples. Partial ITS-1 sequence analysis of the local isolates (PQ900954.1, PQ900955.1, and PQ900956.1) revealed a high degree of similarity to strains from Australia (97%–100%) and to a Chinese isolate (94%).

Conclusion: Strongylosis is an important veterinary disease in local horses, with a 42.3% prevalence and significant association with the month and sex. Phylogenetic analysis revealed significant genetic similarity between strains from Iraq and Australia, highlighting the importance of molecular diagnostics to improve parasite management, prevent the spread of zoonotic diseases, and enhance livestock health.

Keywords: Horses, Phylogenetic tree, Polymerase chain reaction (PCR), Prevalence, Strongylosis

Article info:
Received: 26 Nov 2025
Accepted: 05 Dec 2025
Publish: 01 Mar 2026

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Introduction

Horses are domestic animals that are important to resource-poor populations in both rural and urban areas, providing transportation services in difficult terrain or for military ceremonies, and playing a role in land tillage and cultivation (Andarge et al., 2017; Idoko et al., 2021; Alaba et al., 2022). The global horse population, particularly in Iraq, has declined significantly over the past few decades. This reduction can be attributed to the persistent reliance on traditional methods of horse care and management, such as the use of traditional farms or individual stables for each horse, as well as to inadequate veterinary attention, coupled with the diminishing availability of grasslands and forests (Devkota et al., 2021; Ememe et al., 2024).

Equines exhibit elevated susceptibility to a diverse array of pathogenic gastrointestinal helminths, particularly large strongyles such as *Strongylus vulgaris*, *Strongylus equinus*, and *Strongylus edentatus*, which are the primary causative agents of strongylosis, one of the most important parasitic diseases that affects more than 90% of the equine population (Alhaitami, 2023). Equine large strongyle infections are associated with a range of clinical manifestations, such as reduced productivity, colic, diarrhea, growth impairment, and reproductive problems, and they can lead to pathological changes that include damage to visceral organs, verminous arteritis, and thrombosis, leading to death (Kaur et al., 2019; Kompi et al., 2021). These changes arise not only from the presence of adult parasites within the intestinal tract but also from larval migration through intestinal tissues and systemic organs (Asefa & Dulo, 2017; Berihun et al., 2024). Horses become infected by ingesting feed contaminated with feces containing third-stage larvae (L3). The larvae migrate through the bloodstream to organs such as the liver, spleen, and pancreas, before settling and maturing in the large intestine (Kaur et al., 2019; Ola-Fadunsin et al., 2019; Alaba et al., 2022)

The historical diagnosis of gastrointestinal nematode infections in equines has primarily relied on microscopic identification of nematode eggs or larvae in fecal samples, using flotation techniques and or larval cultures (Bizuayehu & Bedada, 2018). However, these techniques are constrained by difficulties in distinguishing species solely based on egg or larval morphology (Lichtenfels et al., 2008; Jasim & Al-Amery, 2023). Since conventional diagnostic methods have limitations, there is a critical need to adopt molecular approaches, especially polymerase chain reaction (PCR) and sequencing, to accurately identify and distinguish *S. equinus* infection (Bohórquez et al., 2015; Diekmann et al., 2024). Also, this study contributes to the one health approach to equine parasitology by combining molecular surveillance with epidemiological risk-factor analysis, which is crucial for preventing zoonotic spillover and improving livestock health in developing regions.

In Iraq, there is very little information about the prevalence of *Strongylus* spp. despite the significant health risks it poses to horses. This study aimed to evaluate the prevalence of *S. equinus* and the impact of sex, age, and seasonal factors on its incidence in the study area, which had not been previously studied. Besides, this study is the first in Iraq and the region to use molecular diagnostics and phylogenetic analysis to detect *S. equinus* in horses, thereby establishing critical baseline data for future epidemiological studies and control strategies.

Materials and Methods

Study area and sampling

Between January and the end of October 2023, a cross-sectional study was conducted to determine the prevalence of *Strongylus* spp. and to characterize *S. equinus* molecularly. A total of 118 horses (48 females and 70 males) of different ages were randomly selected from stables in the Alkider, Rumathia, and Samawah of Al-Muthanna Governorate, southern Iraq (Figure 1). Sample size (n=118) was calculated based on an expected prevalence of 50% from previous studies, a 95% confidence interval, and a precision ±9%. Five-gram fecal samples were obtained from each horse, either during rectal examination by a plastic glove or from fresh fecal deposits. Each sample was individually stored in a container, clearly labeled with age, sex, and date of collection, and transported to the laboratory in insulated containers with ice packs for direct examination using the flotation method. Then, samples that tested positive by microscopic examination were kept at 4 ˚C for later DNA extraction. Horses were divided into three age categories, namely under four years, four to seven years, and over seven years, based on owner-provided information and dental formulas, as per the methods outlined by Mezgebu et al. (2013).

Coprological examination

To identify *Strongylus* spp. eggs, fecal samples collected from all horses were processed fresh daily via the flotation technique with sheather's sugar solution (prepared

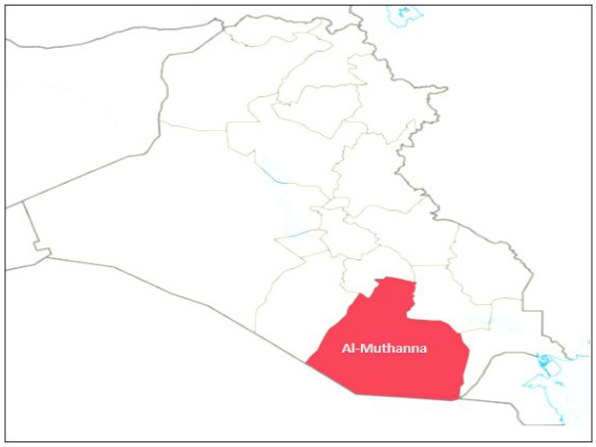

Figure 1. Geographical location of the study areas

with 454 g of sugar, 355 mL of distilled water, and 6 mL of phenol as a preservative; specific gravity 1.27), and the samples were examined under a light microscope at 10x (Alhaitami, 2023). The eggs were identified based on their morphological characteristics, as previously described (Aldelami & Albadrani, 2009).

DNA extraction and PCR

Using the Qiagen DNA stool mini kit (Germany), genomic DNA was extracted from 50 fecal samples of horses that tested positive for *S. equinus* infection based on the flotation technique. Afterward, each eluted DNA sample was measured using a NanoDrop spectrophotometer (Thermo, USA) to determine its concentration and purity by assessing the absorbance ratio at 260/280 nm. Moreover, the integrity of the extracted DNA was assessed via 1% agarose gel electrophoresis supplemented with 0.05% ethidium bromide (Dalimi & Jaffarian, 2024). Partial *S. equinus* sequences from GenBank (Accession No. AJ228250.1) were used to design primers targeting a 220 bp internal transcribed spacer (ITS-1) rDNA fragment for detecting infection in horses, which were ordered from Macrogen (Korea) (Table 1). The PCR amplification mixture for the detection of the ITS-1 region of rDNA included 25 µL of GoTaq® DNA polymerase 2X Master Mix (Promega, USA, and Cat. No. M7122) that contains (Taq polymerase, dNTPs, $MgCl_2$, and buffer), 5 µL DNA template (normalized to 150 ng), 1.5 µL of a 10 µM working stock of each primer (StrongF, StrongR), resulting in a final concentration of 0.3 µM for each primer, and completed the volume to 50 µL with nuclease-free water. The PCR amplification protocol consisted of an initial denaturation at 94 ˚C for 5 minutes, followed by 35 cycles of denaturation at 94 ˚C for 25 seconds, annealing at 56 ˚C (optimized based on primer Tm calculations and gradient PCR) for 25 seconds, and extension at 72 ˚C for 40 seconds. A final extension step at 72 ˚C for 5 minutes was performed. The specificity of the 220 bp product was confirmed by DNA sequencing. After amplification, 5 µL of each PCR product was subjected to electrophoresis on a 1.5% agarose gel and visualized under UV light using a transilluminator (Abdullah & Ali, 2021).

Sequencing and phylogenetic analysis

Three high-quality PCR products with strong band intensity and purity (37, 54, 83) were randomly selected and prepared for bidirectional Sanger sequencing at Macrogen (Korea). The three collected sequences were blasted using online NCBI (BLASTn) software to confirm and collect the aligned sequences. Phylogenetic analysis was performed in MEGA-X v10.0.5 using the Kimura 2-parameter method with 1000 bootstrap replications to ensure tree reliability, with three threads (Hade, 2020). The results of the three local *S. equinus* sequences were submitted to the NCBI-GenBank based on sequence analysis of the ITS-1 region of rDNA with the following accession numbers (PQ900954.1, PQ900955.1, and PQ900956.1).

Statistical analysis

Statistical analyses were performed using SPSS software, version 26.0 (IBM, USA). Prevalence rates were expressed with 95% confidence intervals (CI). The chi-square tests were used to assess associations between infection status and risk factors (age, sex, and season). Variables with $P<0.2$ were further analyzed using multivariate logistic regression to estimate adjusted odds ratios (aOR) with 95% CI. A $P<0.05$ was considered significant.

Table 1. Primer sequences used for detecting *S. equinus*

Primers Name	Sequence (5'→3')	Product Size (bp)	Accession No.
StrongF	TCACGACTTTGTCGGGAAGGTTGGT	220 bp	AJ228250.1
StrongR	GCAATGCTCATCAAGTCTAAAGCTC		

Results

A parasitological examination of equine fecal samples confirmed the presence of *Strongylus* spp. eggs (Figure 2). Epidemiologically, out of the 118 horses examined from January to the end of October 2023, 50 tested positive for *Strongylus* spp., with a prevalence rate of 42.3% based on the flotation technique. Concerning risk factors, infection rates were considerably higher in horses under 4 years of age (57.1%); however, this disparity was not statistically significant (P>0.05) (Table 2). Also, the analysis demonstrated a statistically significant difference in infection rates based on sex, with female horses showing notably higher prevalence (56.2%) compared to male horses (32.8%) (P≤0.05) (Table 2). Moreover, the study identified significant seasonal variations in infection prevalence, with peak rates recorded in March (83.3%) and April (72.2%); these variations were statistically highly significant (P≤0.05) (Table 3).

Genomic DNA was successfully extracted and purified from 50 fecal samples of horses infected with *Strongylus* spp. using the QIAGEN DNA stool mini kit (Germany). DNA concentrations, measured using a Thermo Scientific Nanodrop spectrophotometer (USA), ranged from 52.1 to 211.4 ng/µL, with a purity of 1.8-1.9. Besides, agarose gel electrophoresis confirmed the integrity and purity of the extracted DNA, showing a distinct genomic band without degradation. Furthermore, PCR was performed using specific primers (StrongF, StrongR) to diagnose *S. equinus* infections in horses. The results indicated that 22(44%) out of 50 horses tested positive for *S. equinus* using PCR (Figure 3). Finally, the result of ITS-1 in local *S. equinus* based on phylogenetic analyses were recorded outstanding 100% and 97% identity matching between the current study isolates (PQ900954.1, PQ900955.1., and PQ900956.1) and an Australian strain isolated from horses (AJ228250.1, AJ228248.1), and 94% with Chinese isolate (KP693438.1) (Figure 4).

Discussion

Gastrointestinal parasites, particularly *Strongylus* spp., represent a critical challenge to equine health and productivity in developing nations. These helminths significantly compromise livestock output through impaired digestive efficiency, reduced reproductive performance, and elevated costs associated with therapeutic and prophylactic interventions (Sori et al., 2017; Devkota et al., 2021). Epidemiological analysis revealed that 50 of the 118 horses were positive for *Strongylus* spp. infection with a prevalence rate of 42.3%. This finding is consistent with several prior studies (Hasson, 2014; Oli & Subedi, 2018; Negash et al., 2021; Berihun et al., 2024) but contrasts with others, which documented equine infection rates of 26.5%, 100%, and 71.8%, respectively (Studzińska et al., 2012; Sheferaw & Alemu, 2015; Alhaitami, 2023). The observed variations in *Strongylus* spp. prevalence rates attributed to multiple factors, including differences in sample size, diagnostic meth-

Table 2. Prevalence of *Strongylus* spp. infections in horses by age groups and sex

Factor (Group)		No. of Examined	No. of Infected	Prevalence (%)	Value	95% CI
Sex	Male	70	23	32.8	0.38	0.17, 0.82
	Female	48	27	56.2	1	-
		χ^2=6.2 df=1 P=0.013 n=118				
Age (y)	<4 (young)	21	12	57.1	2.63	0.94, 7.37
	4-7 (adult)	53	23	43.3	1	-
	>7 (old)	44	15	34.1	0.68	0.30, 1.53
		χ^2=2.34 df=2 P=0.31 n=118				

Table 3. Prevalence of *Strongylus* spp. infections in horses occurring during certain months of the year

Months of Years	No. of Examined	No. of Infected	Prevalence (%)	P	95% CI
January 2023	12	3	25	0.198	8.7, 53.2
February	14	5	35.7	0.621	16.3, 61.2
March	12	10	83.3	0.001	54.5, 95.8
April	11	8	72.2	0.012	43.4, 90.3
May	9	5	55.5	0.423	26.6, 81.1
June	12	3	25	0.198	8.7, 53.2
July	10	2	20	0.112	5.7, 51
August	11	2	18.8	0.092	5.3, 47.6
September	13	5	38.4	0.781	17.7, 64.5
October	14	7	50	0.521	26.8, 73.2
		χ^2=20.5 df=9 P=0.015 n=118			

odologies, selection criteria, environmental conditions, pasture management practices, and inconsistent anthelmintic administration (Sheferaw & Alemu, 2015; Alhaitami, 2023; Berihun, 2024).

Furthermore, this study shows that sex possessed a significant impact on the prevalence of infections caused by *Strongylus* spp., which is consistent with previous findings (Hasson, 2014; Alhaitami, 2023; Kebede et al., 2024), and contradicts findings with (Mathewos et al., 2021; Ilić et al., 2023; Berihun et al., 2024), who failed to establish a statistically significant correlation between sex and infection rate. This difference may be attributed to lactation and pregnancy, which can both be immunosuppressive due to stress from cyclical hormonal changes (Alaba et al., 2022; Jasim et al., 2024). Additionally, the epidemiological findings indicated that horses < 4 years of age exhibited the highest prevalence of *Strongylus* spp. infection (57.1%). However, the difference in infection rates among age groups was not statistically significant. These findings are consistent with those of Egbe-Nwiyi et al. (2019) but conflict with those of Alemayehu et al. (2022) and Kebede et al. (2024). The increased incidence of infection in young animals may be attributed to waning maternal immunity, greater exposure to the infective stages of the parasite, and irregular anthelmintic treatment regimens (Singh et al., 2016; Jürgenschellert et al., 2022).

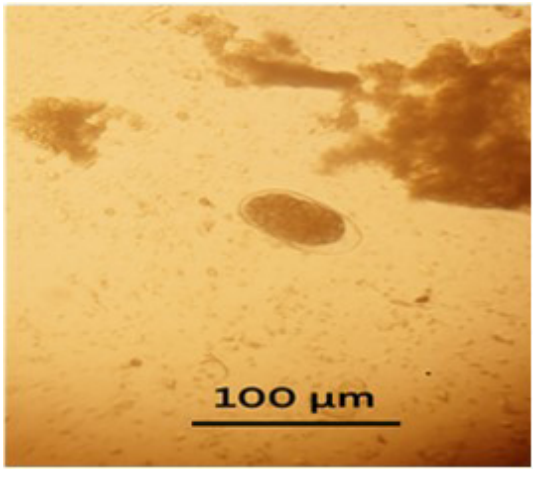

Figure 2. Unstained eggs of *Strongylus* spp. isolated from infected horse feces (scale bar=100 µm)

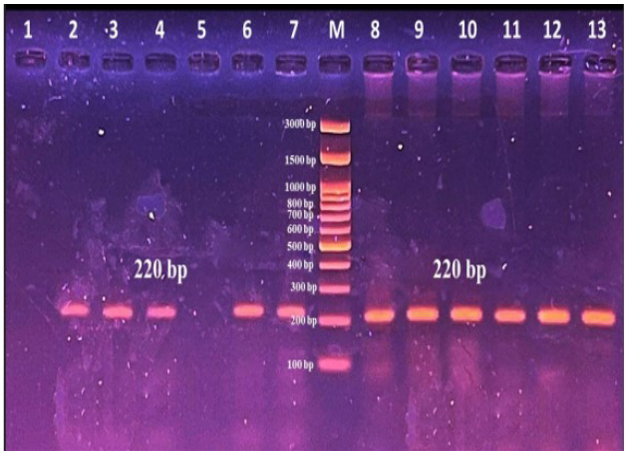

Figure 3. Agarose gel electrophoresis of the PCR amplified DNA from *S. equinus* using primers (StrongF, StrongR)

Note: Lane M: DNA ladder marker (100–1500 bp); Lane 1: Negative control; Lanes 2-4 and 6-12: Positive product at 220 bp that represent samples number (13,17, 23, 33, 35, 37, 54, 61, 73, 83) respectively; Lane 5: Negative isolates; Lane 13: Positive control.

Besides, the study indicated a pronounced seasonal correlation in *Strongylus* spp. infection prevalence, with the highest rate observed in March (83.3%) and the lowest in August (18.8%). These findings correspond with prior studies (Faraj et al., 2007; Hasson, 2014; Singh et al., 2016) and contrast with those of Diekmann et al. (2024), who noted an elevated prevalence of *Strongylus* spp. in July and December. The seasonal fluctuations in *Strongylus* spp. infection rates can be attributed to various interconnected causes, such as the increase in incidence in March, which aligns with the spring birthing season in Iraq, a period characterized by physiological stress and immunosuppression in equines, which increases vulnerability to infection. Simultaneously, ideal climatic conditions, including moderate spring temperatures and humidity, increase embryonation and larval growth, facilitating transmission (Faraj et al., 2007; Singh et al., 2016; Jasim et al., 2024). Additionally, the extended prepatent period of *Strongylus* spp. spanning several months may delay egg excretion in infected equinus until the subsequent grazing season, coinciding with infection peaks with favorable environmental conditions (Studzińska et al., 2012).

This study improved upon earlier methods by combining traditional microscopy with PCR-based molecular diagnostics, demonstrating greater sensitivity compared to. Using PCR to target the ITS-1 region, a 220-bp frag-

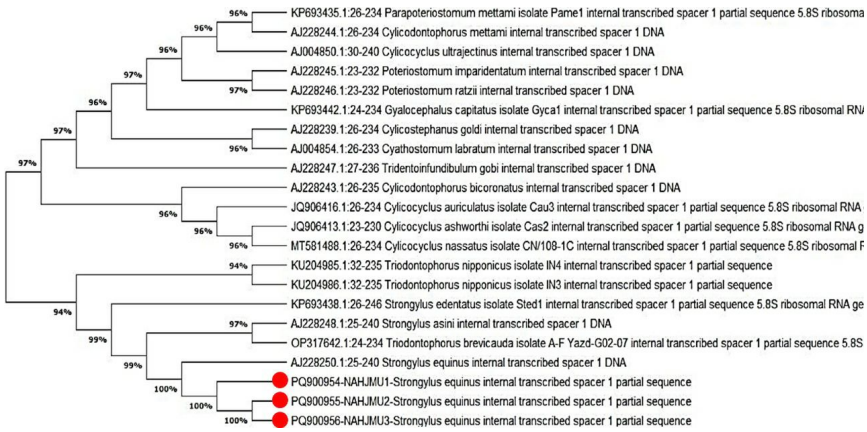

Figure 4. The phylogenetic tree diagram that shows the similarity between the new strains PQ900954.1, PQ900955.1 and PQ900956.1 (red highlights) and other closely related global strains of *S. equinus* according to the sequence of ITS-1 partial sequence gene

Note: The tree was created based on the neighbour- joining method. Numbers represent levels of bootstrap support (%) based on analysis of 1000 replications.

ment was amplified, confirming *S. equinus* in 22(44%) of horse samples. This finding aligns with recent work by Diekmann et al. (2024), which highlighted molecular diagnostics' superior specificity compared to traditional methods, confirming only 1 of 12 morphologically positive samples with PCR. The discrepancies may be due to the inherent limitations of factors in the sensitivity and specificity of morphological diagnostics, such as the developmental stage of the parasite, sample fixation protocols, transportation and preservation conditions, sample size adequacy, and the examiner's expertise in parasitological identification or the morphological overlap among *Strongylus* species (Bowman, 2020; Ghafar et al., 2023).

In addition, the use of ITS-1 as a molecular marker for precise species differentiation of *S. equinus* represents an innovative approach in Iraq. Its reliability compared to traditional diagnostic methods is likely due to the ITS-1 region's significant evolutionary conservation, which renders it an effective molecular marker for taxonomic and phylogenetic research (Halvarsson & Tydén, 2023; Al-Biatee et al., 2024; Ahn et al., 2024). Also, the ITS-1 partial sequence in local *S. equinus*, based on phylogenetic analyses, showed 97%–100% nucleotide sequence identity between the current study isolates (PQ900954.1, PQ900955.1, and PQ900956.1) and an Australian strain isolated from horses, and 94% with a Chinese isolate. The high nucleotide similarity between Iraqi isolates and strains from Australia and China may be due to commercial animal movement—particularly through intermediary countries, rather than direct introduction from those regions or shared ancestral parasite lineages, offering new insights into global transmission patterns of equine parasites (Beltran-Alcrudo et al., 2019). This work is the first study in Iraq to combine molecular diagnostics and phylogenetic analysis for *S. equinus*. These findings provide baseline data for parasite control programs, inform veterinary health policies, and contribute to the global understanding of *Strongylus* spp. genetic diversity.

Conclusion

According to survey data, strongylosis is a major veterinary disease affecting local horses, with a prevalence of 42.3%. It occurs year-round, peaking in spring, and is more common in horses under 4 years of age and in females. Besides, the phylogenetic analysis revealed close genetic similarity among Iraqi, Australian, and Chinese strains, highlighting the evolutionary conservation of the ITS1 region of rDNA as a robust molecular marker for taxonomic and phylogenetic studies. To advance understanding of the parasite's genetic diversity and support One Health goals, the study recommends future initiatives, including whole-genome sequencing of *S. equinus*, expanded surveillance in Iraq, the application of molecular diagnostics to larger equine populations, and an evaluation of anthelmintic resistance.

Ethical Considerations

Compliance with ethical guidelines

Sample collection was performed in compliance with the World Organisation for Animal Health (OIE) animal welfare guidelines and the ARRIVE guidelines. Before collecting the samples, we obtained verbal consent from the horse owners.

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Study design, super vision, review and editing: Hussein Jabar Jasim; Experiments and data collection: Naer Abdulbari Madlool Alkaabawi; Data interpretation, statistical analysis, and writing the original draft: Zahraa Abd Alhammza Abbass and Mohammed Mijbas Mohammed alomari; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors extend their gratitude to the veterinary clinicians and support staff at Al-Muthanna Provincial Veterinary Hospital, as well as the equine breeders, for their invaluable contributions to fieldwork and sample acquisition.

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