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Molecular Conformation of the Equine Redworm, in the large intestine *(Strongylus equinus)* in Iraq.

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ABSTRACT

Background and amis: Large strongyles (large redworms) are the most important gastrointestinal parasites of equids, commonly inhabiting the large intestine and causing various clinical manifestations that may lead to severe disease and death. This study aimed to confirm the natural infection of *Strongylus equinus* in horses and donkeys in Baghdad, Iraq, through morphological and molecular characterization.

Materials and Methods: Samples were collected from necropsied animals during the study period from December 2024, to May, 2025. A total of 65 equids, including horses and donkeys, were examined. Adult parasitic worms were carefully recovered during post-mortem examination and initially identified by to morphological characteristics, particularly buccal capsule. Molecular identification was subsequently performed using (ITS-2) sequence analysis.

Results: The prevalence of *Strongylus equinus* represented 1% of the total *Strongylinae* infections. Recovered specimens were morphologically identified as *S. equinus*. Molecular characterization confirmed species was identity despite the limited availability of *S. equinus* sequence data in GenBank. Phylogenetic analysis showed that the study isolates clustered within the primary clade alongside *S. equinus* (X77808.1) from Australia, *Strongylus asini*, and *Strongylus edentatus* isolates from China. The obtained *S. equinus* sequences were submitted to GenBank under accession numbers PX442255, PX442256, PX442257, and PX442258.

Conclusion: The integration of morphological and molecular identification of adult worms provides reliable species-level diagnosis of *S. equinus* infection in equids, and facilitate possibility of detecting species by eggs and other larval stage.

Introduction

Horses and donkeys are infected by the common GIT Strongyle worm, *Strongylus vulgarus* and also susceptible to infection by nematode species, such as *S. edentatus and S. equinus*, but their impacts are not as severe as the first [1-4]. All these parasites together are referred to as the large red worms, and infections by them as strongylosis, have continued to be one of the most important parasitic diseases in equine world [5].

This has been further proven by the results of numerous epidemiological surveys completed in several countries that gastrointestinal nematode infections are highly prevalent among equines and may lead to significant economic losses by reducing their working capacity, poor body condition and low reproductive performance in addition to the growing problem of the anthelminthic resistance problem [6-8]. The pathology of such parasites is determined by the type and severity of the infection and the overall health condition of a host [9]. A very high morphological stability of *Strongylus* species over long periods of evolution shows that the *Strongylus* species have very conserved genetic characteristics, which has helped them to be effective parasites. Molecular analysis can offer good insights into how these genetic traits evolved, their taxonomic position as well as how they are pathogenic. [10].

Traditionally, the identification of *Strongylus* species has relied on morphological examination of adult worms, focusing on features such as the buccal capsule, spicules, and dorsal gutter. However, this approach can be difficult and unreliable when worms are immature, damaged, or morphologically similar. Modern molecular methods have therefore become essential for accurate species identification and phylogenetic studies. Techniques based on polymerase chain reaction (PCR) and DNA sequencing of ribosomal or mitochondrial markers, allow precise differentiation between closely related *Strongylus* species and provide new tools for epidemiological monitoring and control strategies [11].

In Iraq, the population of equines has declined over recent decades, and few detailed studies have investigated the diversity and distribution of internal parasites in local equine populations [12,13]. Earlier research on equine helminths in Iraq was mainly based on fecal examination, with limited anatomical investigations because of the lack of specialized equine slaughterhouses [14,15]. Consequently, molecular identification and characterization of *Strongylus* species in Iraqi equines remain scarce.

Materials And Methods:

Samples collection:

This research and sampling of animals were conducted in accordance with Institutional Animal Care and Use Committee (**ACUC**) at the University of Baghdad's College of Veterinary Medicine.

There are no experiments done on living animals. Samples were collected from necropsied animals during the study period extending from December 1st, 2024, to May 31st, 2025 at the slaughterhouse of the Al-Zwara Zoo in Baghdad. A total of sixty-five donkeys (55) and horses (10), the animals were purchased from local providers for feeding of the predictor's animals and birds (Fig. 1). The intestines were examined, and parasitic worms were carefully recovered during post-mortem examination All samples were collected under aseptic conditions and were handled according to standard parasitological procedures.

Figure 1: Preparation yard, before processing weekly slaughtering of donkeys and horses.

Post-slaughter necropsy, the intestinal tract was carefully isolated, and the large intestine was separated into its main sections, including the cecum, colon, and rectum. Each intestinal segment was opened sequentially to allow thorough examination. The worms attached to the intestinal wall were directly recovered using a surgical scissor to gently open the intestinal wall, and the worms were carefully detached using blunt forceps (Fig. 2). All collected samples were transferred in a designated sample storage box to the laboratory of the department, where they were examined under a light microscope for further morphological identification and subsequent analyses.

Figure 2: The dark red round worm attached to the intestinal mucosa of the large intestine. (red arrow).

Sample preparation for primary identification

Modified Lactophenol stain

The method carried on a "hot plate" which has been calibrated to 72 C and placed on a 3-mm thick glass depression slide with the cavity filled to excess with glycerin. A careful rotating of the thermometer bulb of a glycerin to determine the proper temperature. The lactophenol formula by mixing, liquid phenol, 209 ml; lactic acid, 177 ml; glycerin, 354 ml; and distilled water, 230 ml. With the lactophenol, a small amount of a 1% cotton blue dye adds to the stock solution. The nematode staining solution was prepared by adding 6 drops of stock 1% cotton blue lactophenol to 50 ml of clear lactophenol [16].

Morphological identification

The collected nematode worms cleared using modified lactophenol stain**.** The identification of worms will be carried out according to morphological characteristics by [17].

Molecular conformation of *Strongylus equinus*

DNA extraction

Four adult worms primarily identified as ***Strongylus equinus*** were preserve in - 20C◦ were applied for DNA extraction using the gSYNC™ Geneaid Biotech Ltd. Germany.

DNA amplification

In Polymerase chain reaction (PCR) for the *S. equinus* amplification, two Forword and reverse primers as in table. 1. were used; forward primer**.**

Table 1: Forword and reverse primers were used by PCR for ITS2 amplification reaction.

Primer	Sequence	Primer sequence 5'- - 3'	Fragment Size	Reference
ITS-2	NC1	ACGTCTGGTTCAGGGTTGTT	170bp	[19]
	NC2	TTAGTTTCTTTTCCTCCGCT		

A thermal cycler (Nexus Gradient; Eppendorf, Germany) was used to conduct the amplification reaction.

The PCR procedure involves 15 minutes at 95C, followed by 40 cycles of 94 C for 15 seconds, 55 C for 30 seconds, and 72C for 30 seconds, as well as melt curve analysis to ensure the PCR result is specific. Using SiZer 100bp DNA Marker as a molecular weight marker, the resultant amplicons were confirmed on a 1.5% agarose gel. Following electroelution, ethidium bromide-stained (0.5 mg/ml) gels were UV transilluminated to reveal the DNA bands.

Sequencing of PCR products and Phylogenetic analysis

Five PCR products are sent for sequence. The result sequences were uploaded to NCBI GenBank to obtain accession numbers. Using BLAST, a basic local alignment search tool, the isolates' nucleotide sequences were matched with reference sequences in NCBI GenBank databases [18,19]. The phylogenetic tree was built to scale, and MEGA 12 was used to quantify branch lengths in terms of the number of substitutions per site. [20].

3. Results and Discussion:

The adult's strongyles worms were collected from the large intestines in examined animals with total infection rate 32.3% (21/65) of strongyles spp. While S. equinus was (4/21)19.04% from the total infected rate. Table 2.

The results were different in a study in Egypt with infection rate of 15.85% [21], when usually, diagnosis for Strongyle worm infection depends on the identification of eggs using traditional method [22]. *Strongyle* eggs, however, are morphologically similar and identification of species is based on larval culture. [23] also in Turkey found with parasite infestation prevalence on draught and workhorses at 2.1% to 95.9% and in donkeys ranged 77.3% to 100%.

Table 2: Infection rates of *Strongylus equinus* from the total *Strongylus* spp. in slaughtered donkeys and horses over the study months.

Month	Total examined animal	infected animal	Total worm infection (%)	*Strongylus equinus*	S.equinus infection (%)
December 2024	10	5	50.00	1(2/5)	20.00
January2025	11	4	36.36	0	0.00
February	12	4	33.33	1(1/4)	25.00
March	10	3	30.00	0	0.00
April	10	2	20.00	0	0.00
May	12	3	25.00	1(1/3)	3.33
Total	65	21	32.3	4(4/21)	19.04

In extremely cold winters and dry summers, the infestation rates tend to be lower. [24] confirmed that the pathological effects of the *Strongylus* spp. parasite on the arteries during winter were 26.4%, compared to 7.1% during other months. The researcher noted severe outbreaks of parasitic diseases in years with mild winters and more humid summers. The adult population was highest in November and lowest in April [25]**.** In donkeys (*Equus asinus*) from Zimbabwe. In Iraq, more than study found that the eggs of these worms remained viable for over the months in the laboratory a month in external environments [26,27].

The larval development of the theses worm requires a prepatent period of more than six months to mature into adults inside an animal's intestines. For this reason, the infective larvae that were ingested by the animal during the last rainy season would not be expected to appear in large numbers in the cecum and colon (the large intestine) before May. Furthermore, the decreasing number of adult worms observed in the gut during January and April indicates that the old worms were dying and being expelled from the body faster than new larvae could mature and replace them [28].

In The diagnosis of *Stronglys* infection is usually made by demonstration of eggs passed in the feces of infected equines, which can be achieved through either larval culture or subsequent microscopic examination. [29].

Morphological Results

The collected Strongylinae species were identified in the primary parasitological examination based on the morphology of the

buccal capsule as *Strongylus vulgaris (Delafondia vulgaris Looss, 1900), Strongylus equinus (S. equinus* Müller, 1784) and. Primary parasitological examination identifies based on the morphology of the buccal capsule; The larvae resembled a robust, inflexible, cylindrical worm with leaf crowns on the front. (Fig. 3,4,5).

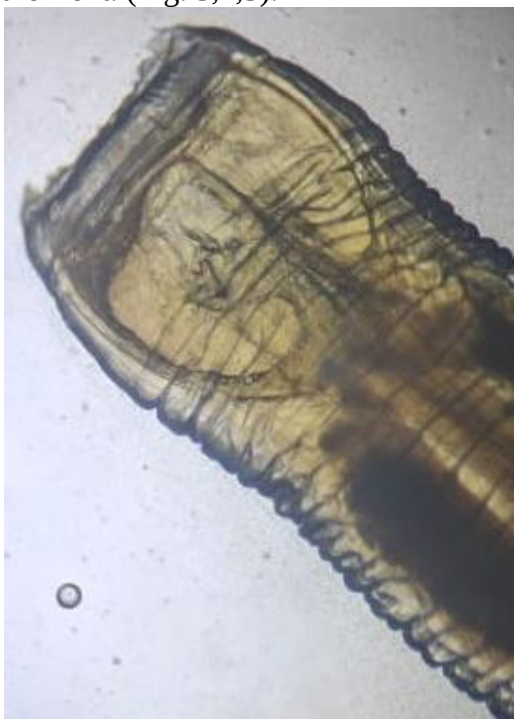

Figure 3: *Strongylus equinus*, buccal capsule and leaf-crown and ventral view of buccal-esophageal region. 40X.

Figure 4: *Strongylus equinus*, lateral view of the male tail and fused spicule tips. 40X.

Figure 5: *Strongylus equinus*, the photograph of the female posterior end with contains the anal pore. 10X.

Molecular results

The DNA amplification of the partial ITS2 for four specimens identified primarily by morphology as *Strongylus equinus*. Successfully, the amplicons with clear band showed in electrophoresis in 1.5% of Agarose with dna fragments ~170bp respectively. (Fig. 6).

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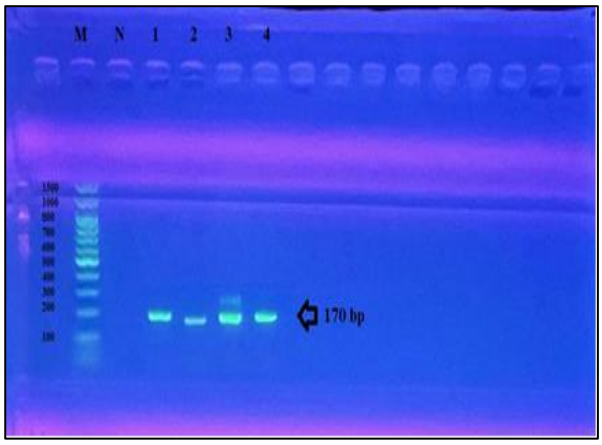

Figure 6: Agarose gel. electrophoresis (1.5%) profile presenting the amplification of *S. equinus*. Lanes: 2, 3, 4 positive amplicons. N:-ve control, M: 100bp ladder. with condition: 1x TAE buffer, 80Vol. for 1:30hrs.

PCR products were successfully sequenced and BLAST (https://blast.ncbi.nlm.nih.gov). The isolates' nucleotide sequences were matched with reference sequences in NCBI GenBank databases. The BLAST results show 98-99% identity and aligned with correspondent reference sequence X77808.1 available at GenBank were phylogenetic analysis shows identity and similarities (Table 3). All the ITS2 partial sequences data for S. equinus were submitted to the GenBank and accession numbers were obtain PX442255, PX442256, PX442257 and PX442258. GenBank http://www.ncbi.nlm.nih.gov. s. For our acknowledge this the first time to sequencing these equine nematodes in Iraq.

Table 3: Identity data with the Nucleotide transition loci compared with the reference sequences.

No.	Type of substitution	Location	Nucleotide	Source	Seq. ID with compare	Seq. ID with submission	Identities
1	Transvertion	92	A\C	*S. equinus*	ID: X77808.1	ID: PX442255.1	99%
	Transvertion	175	A\T				
2	Transvertion	90	C\G	*S. equinus*	ID: X77808.1	ID: PX442256.1	98%
	Transition	91	A\G				
	Transvertion	175	A\T				
3	Transvertion	128	A\C	*S. equinus*	ID: X77808.1	ID: PX442257.1	99%
	Transvertion	175	A\T				
4	Transvertion	175	A\T	*S. equinus*	ID: X77808.1	ID: PX442258.1	99%

Aa Partial ITS2 for four adult specimens were successfully sequenced and aligned with correspondent data available at GenBank.

A phylogenetic tree of the default setting (UPGMA) was constructed (Fig. 7). The numbers in parentheses indicate the evolutionary distance calculated using the Maximum Composite Likelihood method. MEGA.12.

The tree was constructed from eight different isolates including the present study *S. equinus*. Although very limited sequence data of the *S. equinus* was available in the GenBank, the study isolates present in primary clade include the species *S.equinus* X77808.1 from Astrallia, *Strongylus asini* and *S. edentates* from China. The tree out group isolated was with *Haemonchus controtus*. Equine Strongylidae represents a multi-species complex which has been incompletely interpreted with very limited information available regarding taxonomic classification and species diversity [30].

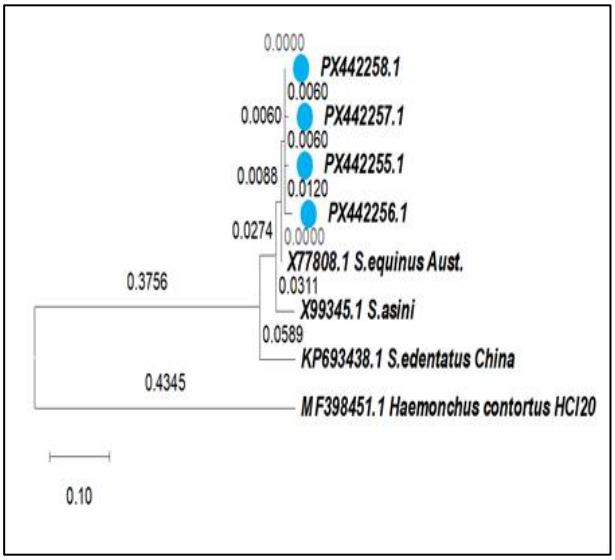

Figure 7: Phylogenetic tree of *Strongylus equinus*. The evolutionary distances were computed using the Maximum Composite Likelihood method. The present study isolates marked with blue dots.

Traditionally, for many nematode species sequencing ITS2 has been considered robust genetic marker. Even, it will not necessarily

give complete illumination of a system [31]. Whereas either ITS1 or ITS2 will typically discriminate species, the use of both regions increases the discriminating power significantly, however, this molecular marker can be for diagnosis of the infection of *S.vulgaris* and other related species in feces by PCR sequencing.[31]. However, even in the presence of variation in the ITS2 region with many species diagnosable because of sequence or length differences alone [32,33]. Moreover, identification of the *S. vulgaris* genotype was a benefit of differentiation between species of Strongylus (*S. vulgaris, S. edentates, and S. equines*).[34].

Conclusion:

The current study reported the prevalence regarding infection rate in donkeys and horses with morphological description of the three large red worms including photography. In addition to a genetic analysis of *S. equinus* isolates represented the first molecular characterization in Iraq. These results also indicated that the ribosomal spacers could be used as genetic markers for species identification of single *Strongylus* species eggs from horse feces as a beneficial method of diagnosis.

Ultimately, morphology-based diagnosis in the adult worm speciation and the molecular identification are of more value in the infected hosts, in addition to the possibility of species by eggs other larval stage.

Conflict of interest statement

The authors declared no conflict of interest.

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Declaration of interests

The authors have no conflicts of interest to declare.

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Publication consent

The authors agree to publication

Data and material availability

All the data obtained were included in the manuscript**.**

Authors contribution:

A.A design the study. H.S and A.A share, samples collection , examination of samples and manuscript writing and editing.

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التأكيد الجزيئي لديدان الخيول الحمراء، في الأمعاء الغليظة المستديرة الخيلية *(Strongylus equinus)* في العراق

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الملخص

المقدمة والاهداف: الديدان الحمراء الكبيرة من أكثر الطفيليات شيوعا في الفصيلة الخيلية، حيث تستوطن الأمعاء الغليظة وتسبب في ظهور علامات سريرية متعددة قد تصل في بعض الحالات الى النفوق. هدفت هذه الدراسة الى التأكيد الجيني للإصابة الطبيعية بديدان المستديرة الخيلية في الخيول والحمير في مدينة بغداد، العراق.

المواد وطرق العمل :. جمعت العينات من الحيوانات خلال مدة الدراسة من 1 كانون الأول 2024 إلى 31 أيار 202. شملت الدراسة فحص 65 حيوانا بواقع 10 الخيول 55 من الحمير، وتم جمع الديدان الطفيلية اثناء الفحص التشريحي بعد ذبح الحيوانات المخصصة لتغذية الحيوانات المفترسة في حديقة حيوانات الزوراء في بغداد.

النتائج :. تم العثور على الطفيليات البالغة أثناء فحص التشريحي للحيوانات المصابة وبلغ معدل انتشار الإصابة ب المستديرة الخيلية 1٪ من إجمالي الإصابات بالديدان التابعة لهذه العائلة. جرى تحديد النوع بصورة أولية اعتمادا على الصفات الشكلية وخاصة الكبسولة الفمية، وتم تأكيد الهوية الجزيئية باستخدام تحليل تسلسل ITS-2، وذالك على الرغم من محدودية توفر البيانات الوراثية لنوع *S. equinus* في GenBank. اظهر تحليل الشجرة الوراثية تموضع عزلات الدراسة الموجودة في الفرع الأولي من الشجرة التطورية مع تقارب من العزلة X77808.1 من Astrallia، وكذالك العزلات Strongylus asini و*S.equinus* المسجلة من الصين. كما تم ايداع عزلات الدراسة في بنك الجينات العالمي، والحصول على أرقام الانضمام PX442255، PX442256، PX442257، PX442258.

الاستنتاجات: أظهرت الدراسة ان الجمع بين التشخيص المورفولوجي المعتمد على الصفات الشكلية للديدان البالغة والتشخيص الجزيئي يوفر وسيلة جيدة للتعرف واكتشاف الأنواع، كما يتيح إمكانية الكشف عن الطفيلي خلال مراحل اليرقات الأخرى والبيوض.

الكلمات المفتاحية: الصفة التشريحية، المستديرات، الشجرة الوراثية، ITS2، بغداد.