Detect of the eggs of *P. equorum* in the feces of horses by traditional method and molecular techniques in Baghdad, Iraq

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**Abstract**

The risk of *P. equorum* infection in horses remains critical. Little studies were conducted to investigate prevalence and molecular analysis of *P. equorum* in Baghdad city, Iraq. In this study traditional detection followed by molecular technique depending on ITS-2 region were used as an attempt to evaluate this nuclear region as a genetic marker to diagnose the parasitic infection. One hundred and thirty-eight fecal samples of horses were collected and examined by direct wet mount smears, floatation method by NaCl. Extraction of genomic material, nested PCR was done followed by phylogenic analysis depending on ITS-2 region performed for the first time in Iraq and genetic substitutions to analyze Iraqi horses. Nested PCR were done after determining the total infection rate 6.52% by conventional technique, including 3.84% in males and 10% in females with significant difference. Highly infection rate 11.42% was recorded in the age group under 2 years and the lower infection rate 4% was found in the age group between 2-4 years with significant difference. The Iraqi isolates were recorded in the Gen Bank under the accession numbers MZ400507.1, MZ400508.1, MZ400509.1, and MZ4005010.1; while, phylogenetic analysis recorded an identity range between 97-100% with China, Australia and USA isolates. *P. equorum* is more distributed in younger horses than elderly in Baghdad city and ITS-2 region is a certain molecular marker for detection *P. equorum* isolates in Iraqi horses.

**Keywords**: *P. equorum* Nested PCR Internal transcribed spacer-2 region Phylogenetic tree, Iraq

**Introduction**

*P. equorum* is a member in the phylum Nematoda, a roundworm which predominantly affecting the health of foals and yearling horses and decreased in elderly with a wide geographical distribution, this parasite starts its life cycle in small intestine of the host during meet and mate between adult worms, then female adult worms produce huge number of eggs which laid in host small intestine then exit with feces or hatches to larval stage which migrate to liver and lung (1-6). Worm burdens can achieve high numbers (7). The infection is manifested clinically as nasal discharge, ill thrift (8), ceased growth, inappetence, rough hair coat, lethargy (9). In severe cases, other signs detected like pneumonia respiratory, bronchial hemorrhage, intestinal impaction, colic or even liver and lungs damage were diagnosis during migration larval phase (1). Many authors detected *P. equorum* as main species of *Parascaris* spp. eggs in feces of horse (10,11). Other study detected *P. univalens* mitochondrial genome with high sequences similarity with *P. equorum* and identical genetic maps (12). Due to scarce of data and the importance of *P. equorum* in horses, this study was designed to estimate the prevalence and molecular detection and some risk factors (sex and age) on the infection rate.
Materials and methods

Ethical approval
Samples were collected and transferred after obtaining approval from the regulations of Iraqi Ministry of Agriculture. The research protocols for the study were approved by the College of Veterinary Medicine, University of Baghdad, and were included in laboratory standards after animal utilization protocol certification No. P. G. 1904 in 2/10/2022.

Samples collection
One hundred and thirty-eight horse fecal samples were collected from different areas in Baghdad city during the period from the beginning of December till the end of October 2022. The fecal samples of 25 g were divided into two parts, the first one 10 g for fecal analysis by using direct wet mount smears and flotation method by using NaCl and the other part 15 g for the molecular study.

Statistical Analysis
The Chi-square was used for assess the significant differences among factors at P≤0.01 (14).

DNA extraction
DNA extraction from *P. equorum* using G- spin DNA extraction kit (Intron, Korea) was done according to the manufacturer’s recommendation. Estimation of Genomic DNA was detected by Nanodrop spectrophotometer to estimate the concentration and its purity at absorbance 260-280 nm.

Primers
Two sets of primers were designed targeting the ITS-2 region; the first PCR run with ITS-2 first run PCR primer; PE1FS'-GCTGCGTTCTTTCATC GAT-3’ and PE1RS'-TTAGTTTCTTT CT CCG CT- 3’ at 400 bp PCR product depend on (15), as well as the second PCR run with ITS-2 second PCR run primer; PE2FS'-AAT GCC ATA TAT GAA ATA TAT ACG-3’ and PE2RS'-TTA GTT TCT TTT CCT CCG CT-3’ at 290 bp PCR product depend on (16).

PCR mix and amplification profile
The DNA was amplified by using nested PCR (first and second) reaction and for gene diagnosis a mixture of the specific interaction components volume include Master mix-Maxime Pre-Mix Kit (i-Taq), template DNA 1.5µl, 1µl forward primer (10pmo); 1µl reverse primer (10pmo) and 16.5µl Free nuclease water to maintain total PCR reaction volume 20 µl.

The optimum thermocycler condition for gene detection was run under the condition (Table 1). Agarose gel Electrophoresis 1.5% had been done to determine DNA pieces and to detect the result of PCR interaction and compare it with marked standard DNA to distinguish the bundles size (17).

<table>
<thead>
<tr>
<th>Condition</th>
<th>1st Temperature</th>
<th>Time</th>
<th>Cycle</th>
<th>2nd Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
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<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
<td></td>
<td>95°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>52°C</td>
<td>35 sec</td>
<td>35</td>
<td>58°C</td>
<td>30 sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
<td>72°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 min</td>
<td>1</td>
<td>72°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
<td></td>
<td>4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequencing and phylogenic tree analysis
Approximately 290 bp Amplicons from four worm samples were sequenced using Sanger sequencing and the resulting sequences were submitted on NCBI and analyzed using MEGA6.0 (18).

Results

Morphology of parasite eggs
The morphological appearance of twenty-five eggs of the parasite from different samples revealed a spherical to sub spherical shape and their measurements were 95-120µm length and 90-110 µm width (Figure 1).

Figure 1: *P. equorum* egg by direct wet mount smears (X10).
Total infection rate

The total infection rate of *P. equorum* in horses was 6.52% (9/138) with a significant difference between males 3.84% and females 10% (Table 2). The rate of infection was the highest 11.42% in age group under 2 years, followed by the age group 4 years old 5.66% and the lower infection rate 4% occurred in the age group between 2-4 years with significant difference (Table 3).

Table 2: Distribution of *P. equorum* infection according to sex of study animals

<table>
<thead>
<tr>
<th>Sex</th>
<th>Total No.</th>
<th>Positive No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>78</td>
<td>3</td>
<td>3.84</td>
</tr>
<tr>
<td>Females</td>
<td>60</td>
<td>6</td>
<td>10.00</td>
</tr>
<tr>
<td>Total</td>
<td>138</td>
<td>9</td>
<td>6.52</td>
</tr>
</tbody>
</table>
\[
\chi^2 = 71.76
\]

Table 3: Prevalence of *P. equorum* infection according to age of horses

<table>
<thead>
<tr>
<th>Age (Year)</th>
<th>Total No.</th>
<th>Positive No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2</td>
<td>35</td>
<td>4</td>
<td>11.42</td>
</tr>
<tr>
<td>&gt;2-4</td>
<td>50</td>
<td>2</td>
<td>4.00</td>
</tr>
<tr>
<td>&gt; 4</td>
<td>53</td>
<td>3</td>
<td>5.66</td>
</tr>
<tr>
<td>Total</td>
<td>138</td>
<td>9</td>
<td>6.52</td>
</tr>
</tbody>
</table>
\[
\chi^2 = 52.39
\]

Molecular analysis

Molecular findings detected that DNA purity and concentration were 1.8 and 300 ng/µl respectively. Nested PCR technique was done for 10 randomly collected samples, using two primers for ITS-2 region. Results of amplification of 5 samples with 400 bp in first PCR run and 290 bp in second run were showed by electrophoresis of 1.5% agarose (Figures 2 and 3).

Four positive local isolates were sequencing and submitted in NCBI with accession numbers MZ400507.1; MZ400508.1; MZ400509.1 and MZ4005010.1 then phylogenetic analysis indicated that Iraqi isolates were closely to Australia, China and USA isolates with very low genetic diversity at 0.0035 (Figure 4).

Genetic substitutions for Iraqi isolate compared with China isolate ID: MK209647.1. Results indicated transition in location 742 A/G with isolate MZ400507.1. Transition and trans-version with isolate MZ4005010 in the locations 495 C/T,501G/T,709 T/C and 719 T/C (Transition) and 550 A/C,692 G/C and 707 A/T (Trans-version) while other isolates showed no genetic substitution (Table 4).
Table 4: *P. equorum* ITS-2 region substitutions Iraqi isolates

<table>
<thead>
<tr>
<th>No.</th>
<th>Type</th>
<th>Location</th>
<th>Nucleotide</th>
<th>Sequence ID</th>
<th>Source</th>
<th>Identify</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transition</td>
<td>742</td>
<td>A/G</td>
<td>MK209647.1</td>
<td><em>P. equorum</em></td>
<td>99%</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>MK209647.1</td>
<td><em>P. equorum</em></td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>MK209647.1</td>
<td><em>P. equorum</em></td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>Transition</td>
<td>719</td>
<td>T/C</td>
<td>MK209647.1</td>
<td><em>P. equorum</em></td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>Transition</td>
<td>709</td>
<td>T/C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trans-vert</td>
<td>707</td>
<td>A/T</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Trans-vert</td>
<td>692</td>
<td>G/C</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Trans-vert</td>
<td>550</td>
<td>A/C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transition</td>
<td>501</td>
<td>G/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transition</td>
<td>495</td>
<td>C/T</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Discussion

*P. equorum* is a nematode roundworm of young horses with wide geographical range (19). This study describes the results of detection of the infection in horses in Baghdad city by using microscopic examination; direct wet mount smears and flotation methods of fecal samples and molecular confirmative diagnosis by Nested PCR. The results of the present study show spherical to sub spherical shape of *P. equorum* eggs and their measurements between 104×98.2 μm, length and width respectively. This agreed with Zajac and Conboy (20) who found that eggs of the parasite spherical in shape with diameter 90-100 μm. The total infection rate of *P. equorum* was 6.52%. This result disagreed with previous studies in some countries such as Lind and Christensson (6) who found 48% positive eggs samples for *P. equorum*. Also, Romero et al. (21) analyzed fecal samples by coproparasitological concentration-flotation technique of 218 horses found 11.93% were infected with *Parascaris spp*.

According to age, *P. equorum* infections occur commonly in foals, yearlings and seldom harbored in horses older than 4 years (6). Regarding gender, Nielsen et al. (13) reported that the equine ascariid occurred predominantly in foals and this is in agreement with the results of the present study. Due to resistant of eggs to the environmental conditions, this leads to increasing the infection rate of *P. equorum* and this agreed with Reinemeyer (9) who indicated that the survival of larvae eggs up to 10 years and the highly development of infective stage (3rd larvae) within 10 days potentiate the survival of the parasite and increased the infection in the field (22). Breed with place of origin were significantly associated with helminth infection (21). Nielsen et al. (13) stated that no studies in naturally infected horses have detected occurrence of the two *Parascaris* species.

The molecular results of the present study indicated that ITS-2 region was good genetic marker for detecting different nematode parasites, *P. equorum* for the first time in Iraq, that agreement with other studies which detected parasites infection and manifested dependent of genetic marker on ITS region as good molecular marker (23-30), due to difficulty of diagnosis of parasite by traditional methods (30). The four sequences were fell into same cluster and aligned with other 10 sequences of ITS-2 region isolate from different countries depend on final aligned sequence which closely related taxa and only one Iraqi isolate was located outside main cluster as a highly supported sister cluster rooted in a basal position to the main group. Phylogenetic tree-maximum-likelihood was generated from final alignment sequences and showed the main four Iraqi isolate of *P. equorum* and eight isolates of *P. equorum* identified in China and one from each Australia and USA. Most isolates of Iraq connected with main clades with (97-100%) identity, coinciding with other studies (16,23,31). Many studies used phylogenetic tree comparisons with molecular parasites genome revealed high genetic similarities among local isolates and the globally registered sequences (31-39). Molecular detection and Phylogeny one of identification methods used in many studies (40-43).

Comparing local ITS-2 region sequences of *P. equorum* with accession MK209647.1, results showed many genetic substitutions (transition and trans-version) which indicated that Nested-PCR technique followed by phylogenic and substitution analysis to ribosomal rDNA ITS-2 region was suggested for estimation of genetic diversity which considered the main genetic line study to detect genetic homology (23,24).

Conclusion

*P. equorum* occurs in a high percentage in horses at Baghdad city. ITS-2 region is a good molecular marker for diagnosis the infection of *P. equorum*. Molecular detection with nested PCR flowed by phylogenic tree analysis were very useful to detection and diagnosis parasite *P. equorum* in horses.

Acknowledgments

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Conflict of interests

The author has no conflict of interest.

References


الكشف عن بيوس طفيلي إسكارس الخيول في عينات براز الخيول باستخدام الطرق التقليدية والجزيئية في مدينة بغداد، العراق

الخلاصة

يؤدي الخمج بديدان إسكارس الخيول إلى حدوث أذى في الخيول. توجد دراسات جزيئية قليلة لتحديد نسبة انتشار الطفيلي في مدينة بغداد، العراق. اعتمدت الدراسة الحالية على الطرق التقليدية لتشخيص الطفيلي ويتبعه الكشف الجزيئي لتأكيد الاصابة. نسيج داخلي -2 كمحاولة لتحديدها كعلامة وراثية لتشخيص العدوى بالطفيلي. تم جمع مادة وحيدة وثلاثين عينة براز من الخيول مع تحديد عمر وحجم الحيوانات. تم فحص عينات البراز بعد مسحات مباشرة، طريقة التطبيف بواسطة كورهير الصوديوم. تم استخلاص المادة الوراثية ومعالجة البلاست يتبعها تفاعل البلمرة المتسلسل المتداخل. تم إجراء تفاعل البلمرة المتسلسل المتداخل بعد تحديد معدل الإصابة الكلي 15.12% بالطرائق التقليدية. وسجلت أقل نسبة إصابة 7.84% في الذكور والأعلى 19.10% في الإناث مع اختلاف معنوي. تم تسجيل ارتفاع معدل إصابة 15.74% في القناة العميقة أقل من عامين، بينما وجد أقل معدل الإصابة 4.71% في القناة العميقة بين 2 - 5 سنوات مع وجود فرق معنوي. تم تسجيل العينات المحلية في بنك الجينات العالمي. بينما سجل مجمل شجرة المجرة الوراثية تطابق 100% مع عزلات الصين وأستراليا والولايات المتحدة الأمريكية. يعتقد أن طفيلي إسكارس الخيل الأكثر انتشاراً في الخيول ذات الأعمار الصغيرة في مدينة بغداد، ويعود فاصل نسخ داخلي -2 منطقة وراثية يمكن استخدامها بشكل مؤكد في تشخيص الخمج بالطفيلي في الخيول.