

CONVENTIONAL AND MOLECULAR STUDY OF *Babesia* spp. OF NATURAL INFECTION IN DRAGGING HORSES AT SOME AREAS OF BAGHDAD CITY, IRAQ

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ABSTRACT

The present study was planned to investigate equine babesiosis in dragging horses in Baghdad city, Iraq by using microscopical and molecular (PCR) techniques. 150 blood samples of horses examined for *Theileria equi* and *Babesia caballi*. 16.66% (25/150) were positive by microscopic examination. No significant difference was observed in infection rates between male and female horses and among different age groups. The result showed that PCR method has high rate of infection 36% (9/25). Nine positive PCR products were sequenced and deposited in Genbank data base for first time in Iraq, phylogenetic analysis demonstrated that 5 sequences belongs to *T. equi* (MK350319, MK346272, MK346273, MK346274 and MK36275), while 4 sequence (MK346276, MK346277, MK346278 and MK350318) belongs to *B. caballi*, and mounted a low genetic variation 0.035 and 0.05 respectively, among other comparison isolates. In conclusion PCR technique followed by phylogenetic tree analysis a reliable methods for epidemiological, diagnosis and identification of genetic variants studies.

Key word: molecular techniques, *Theileria equi*, *Babesia caballi* , horses, Iraq.

مجلة العلوم الزراعية العراقية -2019: 50(3):909-915

الدراسة التقليدية و الجزيئية لطفيلي *Babesia spp.* للعدوى الطبيعية في خيول الجر في بعض مناطق مدينة بغداد ، العراق

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المستخلص

صممت الدراسة الحالية للتحري عن داء الكمثرات في خيول الجر في مدينة بغداد، العراق. باستخدام الفحص المجهرى و الفحص الجزيئى (PCR). فحصت 150 عينة من دم الخيول بالفحص المجهرى وكانت نسبة الاصابة الكلية لطفيلي *Babesia spp.* 16.66 % (25/150). ولم يسجل فرق معنوي في نسب الإصابة بين الذكور والإناث وبين الفئات العمرية المختلفة. أظهرت النتائج الفحص بتقنية تفاعل سلسلة البلمرة نسبة اصابة بلغت 36 % (9/25). تم ارسال تسع عزلات موجبة باستخدام الفحص الجزيئى و إيداعها في قاعدة بيانات بنك الجينات العالمى، حيث أظهرت نتائج شجرة التطور الوراثى أن 5 عزلات تنتمي إلى *Theileri. equi* سجلت بالارقام (MK350319, MK346272, MK346273, MK346274 , MK36275) و 4 عزلات تنتمي إلى *Babesia caballi* سجلت بالارقام (MK346276, MK346277, MK346278 and MK350318) كما وظهرت النتائج تبايناً وراثياً على مستوى القواعد النيوكليوتيدات منخفضاً 0.035 و 0.05 على التوالي مقارنة بالعزلات الأخرى. نستخلص ان تقنية PCR وتحليل شجرة التطور الوراثى أساليب موثوقة للدراسات الوبائية وللتشخيص ولتحديد المتغيرات الجينية.

كلمات مفتاحية: الكمثرات، سلسلة البلمرة، بنك الجينات.

INTRODUCTION

Equine babesiosis as the most important blood parasitic tick-borne disease affecting equids (horse, pony, donkey, and mule), it is produced by *Babesia* spp. (*B. caballi* and *Theileria equi*) protozoan (12,13). *Babesia* species are transmitted by tick species belong to the genera *Hyalomma*, *Rhipicephalus* and *Boophilus* (27). The disease of equine babesiosis characterized is by fever, pale mucous membranes, icterus and hemoglobinuria leading to eventual loss of condition of the animal (23, 28). Causative agent of equine babesiosis can be detected by microscope; however, this technique has the disadvantage of being having low sensitivity, mostly in horses with low parasitemia (14). Diagnosis of piroplasms using molecular technique is very necessary for *B. caballi* and *T. equi*, by PCR especial employment which overcome conventional and serological techniques problems, since it was reliable diagnosis method for cryptic species (2, 17). Aimed of this study is *Babesia* spp detection in horses by using molecular technique followed by sequencing and phylogenetic tree analysis of the 18s rRNA gene.

MATERIALS AND METHODES

Samples collection

150 blood samples were collected randomly from dragging horses in some areas of Baghdad city, during from January to July 2018, blood samples was taken from the jugular vein from horses of different breed, ages and of sexes, 5 ml blood samples were collected from each animal and was divided into two parts, the first (2 ml) of the blood used immediately for blood smears stained with Giemsa stain, and the rest (2-3ml) of blood was collected in EDTA tube, for DNA extraction. All samples were transport in ice

box to the laboratory – Veterinary Medicine College- University of Baghdad.

Microscopic examination

Thin smears of the blood smears were prepared from the first part 2 ml of blood samples collected from animals according to (9).

Molecular diagnosis method isolation of genomic DNA from whole blood

The second part of blood (2-3 ml) collected in EDTA tube is used to isolate the genomic DNA from the whole blood samples. 25 blood samples (collected randomly from 150 blood sample examined previously by microscope) used in DNA extraction purpose according to commercial purification method described by G- spin DNA extraction kit cat.no. 17045 (INTRON biotechnology, Korya). A final DNA sample of 50 µl was eluted and stored at -20 °C until analyzed by PCR. Estimation of the DNA Concentration and Purity carried out according to a method presented by Sambrook *et al.* (20). Red save stained 0.8% agarose gel applied for detected DNA quality and integrity (11). PCR component performed using two pairs of specific oligonucleotide primers against the gene (18s rRNA) of *Babesia* spp. in table (1) first primer mentioned by Casati *et al.* (4) and second primer described by Oosthuizen *et al.* (16). PCR amplification consist from PCR master mix with a final reaction volume of (25µL), Master Mix (5µl), DNA template(1.5µl), (1µl) of each primer, then (16. 5µl) D.W. PCR conditions start with initial denaturation(94°C for 5 min), denaturation(94°C 45min), A 35 cycle of annealing 58°C for first primer and 60°C for second primer for 45 min, extension(72°C for 45min) with final extension of(72°C for 5 min).

Table 1. The primer with their sequence and product size

Primer	Sequence	Tm(°C)	Product size
First forward	5'-GTCTTGTAATTGGAATGATGG - 3'	54.3	411-452bp
First reverse	5'-TAGTTTATGGTTAGGACTACG- 3'	49.4	
Second forward	5'-GGGTTCGATTCCGGAGAGGG - 3'	60.3	752 bp
Second reverse	5'-CCCGTGTGAGTCAAATTAAGCCG-3'	60.1	

Sequencing and phylogenetic analysis

PCR products purified using (INTRON) kit and analyzer (Macrogen) using terminator cycle sequencing and BLAST analysis database (<http://blast.ncbi.nlm.nih.gov>), edited

with (Mega 6) then analyses by (Neighbour Joining Method).

Statistical Analysis: All data were subjected to Statistical Analysis System- SAS

(2012)(21) . The differences factors were assessed by chi square test.

RESULTS AND DISCUSSION

Out of 150 blood samples of horses examined for *T.equi* and *B.caballi*, 16.66% (25/150) were positive by microscopic examination. The microscopic examination of the blood smear revealed that the *Babesia* spp. appear in many shapes (round shape and classic tetrad-forms inside the erythrocyte). The prevalence in males was 14.28% (10/70), while in females was 18.75% (15/80). No significant difference

was observed in prevalence of *Babesia* spp. infection between female and male, which means female and male are equally affected with *Babesia* species irrespective of sex. Regarding to age the results revealed that age groups recorded a percentage 15%(9/60) in ages equal or less than four year and 17.77%(16/90) in the ages more than four years significant difference in prevalence was not observed in two age group (Table 2,3)(Figure1).

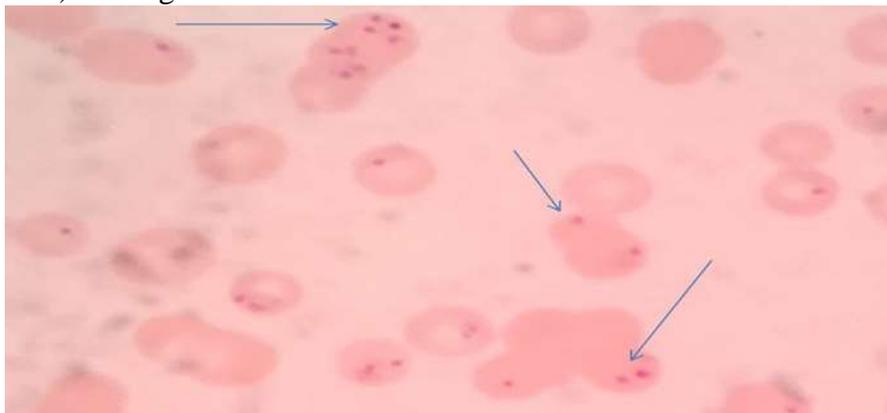


Figure 1. Blood smear stained with giemsa of infected horse with *Babesia* spp.: Round shape and classic tetrad-forms, (maltase cross) and inside the RBC (blue arrow) (X100).

Table 2. Rate of *Babesia* spp, infection according to horses sexes

Sex	No. animal examined	No. animal infected	%
Male	70	10	14.28
Female	80	15	18.75
Total	150	25	16.66
Chi-Square (χ^2)	---	---	0.902 NS

NS: Non-Significant.

Table 3. Rate of *Babesia* spp, infection according to horses age.

Age/year	No. animal examined	No. animal infected	%
< 4 years	60	9	15.00
\geq 4 years	90	16	17.77
Total	150	25	16.66
Chi-Square (χ^2)	---	---	0.894 NS

NS: Non-Significant.

Present study revealed 16.66% rate of Babesiosis infection according to the microscopic examination, and this indicate there is *Babesia* infection distribution on site of study. Present study finding was close accordance with Sumbria *et al.*, (25) in Punjab, in India 14.14% was prevalence rate of *T. equi* and *B. caballi* infection. Also with studies of Mahmoud *et al.* (9) and Ibrahim *et al.* (8) they

reported the prevalence of babesiosis in Egypt was 13.6% and 18% respectively. The studies from other countries revealed high prevalence rate like 20.1% and 41.7% was reported from Niger state by (18, 19). Fluctuation in the prevalence rates might be due to different sample number and variation of environmental conditions that affect both parasites and vectors. In this study no differences were

observed between horse age and sex with the rate of infection, which is consistent with findings by (10,19,24). These result might be due to high numbers of ticks in this area and continuous exposure of horses to infected ticks (18).

Molecular Assay Results

Twenty-five blood sample collected randomly from total 150 microscopically examined, were subjected to extracted genomic DNA, the result obtained high DNA concentration range between (100-140 ng/μl) with purity (1.5-1.6) as showed in (Figure 2).

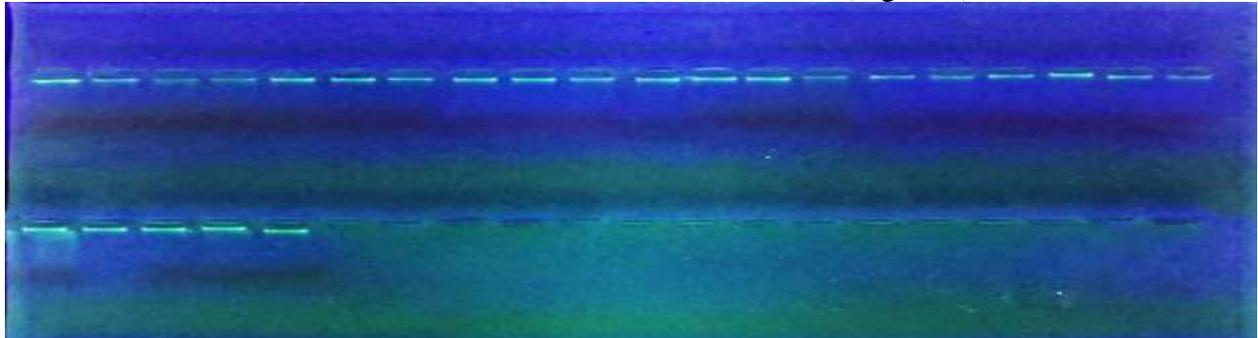


Figure 2. Gel electrophoresis of genomic DNA extraction from blood, Voltage 60, Gel concentration: 0.8 % agarose, Time: 1h

The PCR results revealed that 5 samples found positive for *Babesia* spp. with 441 bp using first primer and 4 samples found positive for

Babesia spp. with (751) bp using second primer as demonstrated in (Figure 3,4).

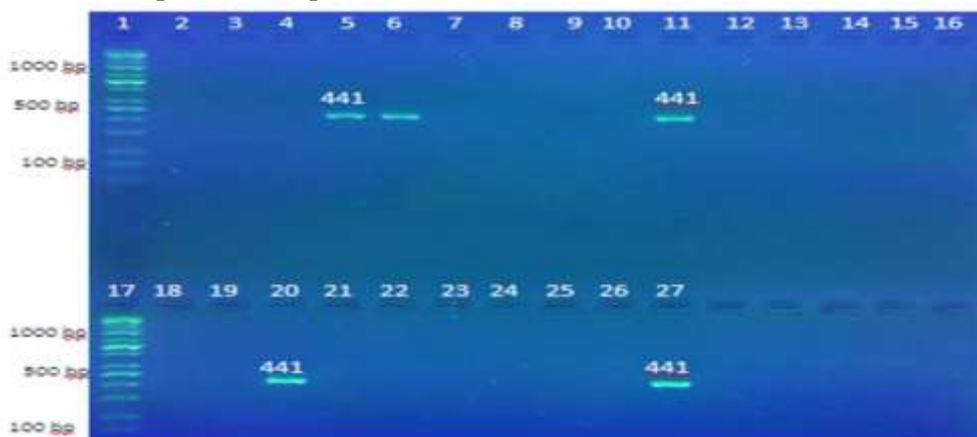


Figure 3. lines 1, 17: molecular marker (100bp); lines 5, 6, 11, 20 and 27 are positive *Babesia* spp. samples (441 bp); other lines were negative samples. The product was electrophoresis on 1.5% agarose at 75 volt for 1.5 hours



Figure 4. lines 1, 17: molecular marker (100bp); lines 3, 7, 15 and 22 are positive *Babesia* spp. samples (751 bp); other lines were negative samples. The product was electrophoresis on 1.5% agarose at 5 volt/cm². 1.5 hours

In total, 9 PCR products were sequenced and deposited in GeneBank in accession numbers; MK350319, MK346272, MK346273, MK346274, MK36275, MK346276, MK346277, MK346278 and MK350318

Phylogenetic Analysis

The result of sequencing revealed that 5 PCR products were (MK350319, MK346272, MK346273, MK346274 and MK36275) belongs to *T. equi* isolates, while (MK346276, MK346277, MK346278 and MK350318)

belongs to *B. caballi*. Comparative sequence analysis mounted a low genetic variation (0.035) among all comparison isolates and revealed 99 –100%. Sequences result showed that MK36275 Iraqi isolate was 100% identity to Spain isolate, MK346273 and MK346274 Iraqi isolates showed 100% identity between each other, while MK350319 and MK346272 Iraqi isolates showed 99% identity among all isolates as clarified in (Figure 5).

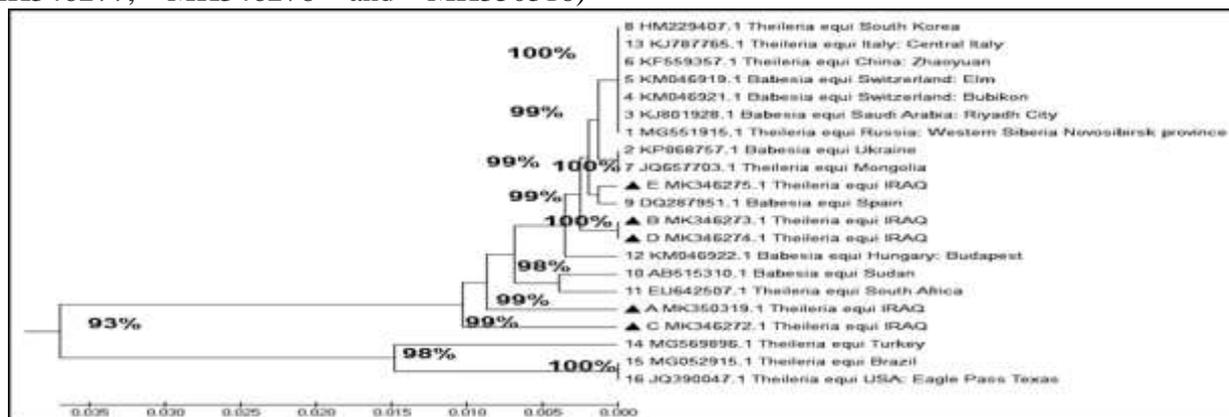


Figure 5. Theileria equi Phylogenetic analysis.

The phylogenetic relationships mounted a low genetic variation (0.05) among all comparison isolates of the *B. caballi* of Iraqi isolates and other *B. caballi* isolates published achieved from GenBank showed 100% identity among

MK346276, MK346277, MK346278 and MK350318 Iraqi isolates and clarified that South Africa and Spain are more close to them as demonstrated in (Figure6).

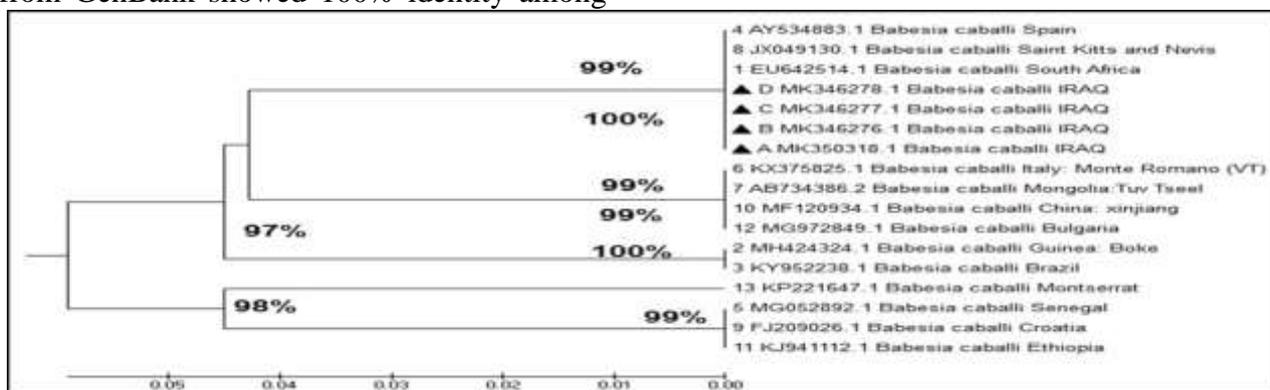


Figure 6. Babesia caballi phylogenetic tree.

Molecular blood examination for study group animals using PCR reaction depending on oligonucleotide primers specific for Equine piroplasmosis revealed that 36% were positive

for the presence of piroplasmid DNA, while traditional method depending on Giemsa stain were found 8% positive, with significant (P<0.01) as showed in (Table4).

Table 4. Comparison between the results of microscopic examination and molecular method and differentiate between species

Technique	No. of blood samples	No. infected animals	%	Chi-Square (χ^2)	<i>B. caballi</i> Infection	<i>T. equi</i> Infection
Microscopic examination	25	2	8	8.263 **	0	0
PCR	25	9	36		4	5

** (P<0.01).

The result of microscopic examination comparison with PCR method revealed that PCR method has high rate of infection than microscopic examination and that results agrees with (1, 13). The current study revealed low sensitivity of Giemsa stained blood smear examination test to detect few level of parasitemia when compared to the PCR technique. Our molecular assay study proved using two different primers to diagnosis of *B. caballi* and *T. equi* in Equine, and can also be used to detect these parasites in animals suspected carrier and that deal with El-Naga and Barghash, (6) results indicated that molecular method could detected DNA of *Theileria* and *Babesia* subspecies present and it an accurate technique . Another previous molecular biological assay studies improved many genetic haemoparasites detection (3,15), Sibeko *et al.* (22) indicted high sensitivity and specificity of PCR to detected *Babesia* and *Theileria* spp. than serological test.

The study results demonstrated *B. caballi* and *T. equi* occurrence and circulation in Equine, and clarified that the ribosomal RNA genes stability makes them an attractive target for species discrimination of this parasite by molecular assay. Additionally, PCR technique followed by phylogenetic tree analysis good methods for detection and identification of genetic variants.

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