Microscopic identification, molecular and phylogenetic analysis of *Babesia* species in buffalo from slaughter house in Al-Najaf city of Iraq

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Abstract

Babesia is one of hemoprotozoan parasite transmitted by arthropod vectors which responsible for causing of Babesiosis disease in bovine worldwide. The present study was designed for microscopic identification, molecular, and phylogenetic analysis of *Babesia* species in buffalo from slaughter house in Al-Najaf city of Iraq. The study performed in three months of summer season (August into September 2017) and animals ages and sex were included in this study. The direct microscopic prevalence results were show highest prevalence of haemoprotozoa prevalence at *Babesia* sp. 45.74%. The prevalence of *Babesia* sp. related to animal sex, were show in male 43.48% and female was 52%, with non-significant differences. The Prevalence of *Babesia* sp. related to age were show 12.50%, 92.86% and 30% in young, adult and old age respectively with significant differences ($P<0.05$). The prevalence of *Babesia* sp. related to month of study were show. 28.57%, 62.50% and 42.86 in August, September and October respectively and with non-significant differences. Molecular study results were based on PCR and DNA sequencing method by phylogenetic tree analysis (MEGA 6.0) and NCBI-BLAST Homology Sequence Identity to differentiation *Babesia* species typing. The *Babesia* species prevalence results were show identified two *Babesia* species, high prevalence of *Babesia* bovis (38.30%) were closed related to NCBI-Blast *Babesia* bovis (HQ264126.1) with homology sequence identity 97-100% and *Babesia bigemina* 7.45% were closed related to NCBI-Blast *Babesia bigemina* (KU206291.1) with homology sequence identity 95-99%, then 43 *Babesia* species includes (*B. bovis* and *B. bigemina*) were submitted into NCBI-Genbank and provided accession numbers (MH503811-MH503853). In conclusion, this study concluded that Phylogenetic tree and homology sequences identity was show accurate in differentiation of *Babesia* species, and these species can be isolated at from local water buffalo from slaughter house in Al-Najaf city, of Iraq.

Keyword: Babesia, PCR, Phylogenetic tree, Buffalo.
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التشخيص المجهري والتحليل الجزيئي والسلالة الوراثية لأنواع طفيلي البابيزيا في الجاموس من مجزرة مدينة النجف في العراق

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الخلاصة

طفيلي البابيزيا هو من الطفيليات أوالي المحمولة بالدم تنتقل بواسطة الحشرات مفصلية الأرجل ومسؤولة عن وحدوث إصابات حادة كمرض في الأبقار في جميع أنحاء العالم. صممت الدراسة الحالية للتحري عن طفيلي البابيزيا من نماذج دم الجاموس للمرة الأولى في العراق. باستخدام طرق الفحص المجهري المباشر والطرق التحليل الجزيئي كفحص البي سي ار وتسلسل تتابع الحمض النووي. أنجزت الدراسة في مدة ثلاثة أشهر في موسم الصيف خلال (شهر أب إلى شهر تشرين الأول لسنة ٢٠١٧). كذلك تضمنت الدراسة علاقة عمر الحيوان وجنسه. أظهرت نتائج نسب الفحص المجهري المباشر وجود نسبة انتشار عالية لأنواع طفيلي البابيزيا بنسبة ٤٥,٧٤% وكانت علاقة نسبة انتشار الطفيليات مع الجنس في طفيلي البابيزيا في الذكور بنسبة ٤٣,٤٨% وفي الإناث بنسبة ٥٢%. أظهرت نتائج الدراسة

حسب العمر نسبة انتشار طفيلي البابيزيا بنسبة ١٢,٥٠% في الصغيرة و٩٢,٨٦% في البالغة و ٣٠% في الحيوانات الكبيرة مع وجود فروقات معنوية بنسبة احتمال اقل من ٠,٠٥. أظهرت نتائج الدراسة حسب الفترة نسبة انتشار طفيلي البابيزيا بنسبة ٢٨,٥٧% في شهر اب و٦٢,٥٠% في شهر أيلول و ٤٢,٨٦% في شهر تشرين الأول مع عدم وجود فروقات معنوية. وأظهرت نتائج التحليل الجزيئي وجود نوعيين لطفيلي البابيزيا وهي طفيلي البابيزيا بوفس بنسبة ٣٨,٣٠% بعلاقة جينية متقاربة لطفيلي طفيلي البابيزيا البقرية المسجل (HQ264126.1) مع نسبة تطابق بلغت ٩٧-١٠٠%. وطفيلي البابيزيا التوأمية بنسبة ٧,٤٥%. بعلاقة جينية متقاربة لطفيلي طفيلي البابيزيا بايجيمنا المسجل (KU206291.1) مع نسبة تطابق بلغت ٩٥-٩٩%. بعد ذلك ٤٣ نوع من أنواع البابيزيا المفرقة سجلت في موقع بنك الجينات للحصول على أرقام تسجيلية من (MH503811-MH503853). في الاستنتاج، استنتجت الدراسة بان تحليل الشجرة الوراثية وفحص التطابق بين القواعد النيتروجينية يعطي تفريق دقيق لأنواع طفيلي البابيزيا والتي يمكن أن تعزل بنسب عالية من جاموس الماء المحلية في محافظة النجف.

Introduction

Babesia is most important Haemoprotozoa parasites of cattle and buffaloes (1). These parasites cause diseases characterized by infected of erythrocytes and caused severe anemia and highly losses in livestock industry throughout the world (2). *Babesia* protozoal parasites are tick borne protozoa and consider economically important disease because of direct losses of milk and meat production (3). However, their outbreak in exotic and crossbred cattle is mostly reported during the hot and humid months of the year (4). Babesiosis is recoded as the most ubiquitous and widespread haemoprotozoa in the world based on numbers and distribution of species in animals, other Haemoprotozoa has recorded as month wise infection of Theileriosis, Anaplasmosis and Trypnasomiasis in crossbred cattle and buffaloes (5). Babesiosis is a tick-borne disease of cattle caused by the protozoan parasites of the genus *Babesia*. The principal species of *Babesia* that cause babesiosis in cattle and buffalo are: *Babesia bovis*, *Babesia bigemina* and *Babesia divergens* (6). *B. bovis* and *B. bigemina* which affected cattle, water buffalo (*Bubalus bubalis*) and African buffalo (*Syncerus caffer*). While *B. divergens* mostly affected cattle and reindeer (Rangifer tarandus) (7). There are less survey studies about distribution of *Babesia* species in buffalo of Al-Najar city slaughter house. Therefore, our study designed to microscopic identification, molecular and, and phylogenetic analysis of *Babesia* species in buffalo from slaughter house in Al-Najaf city of Iraq.

Materials and methods

Blood samples collections

A 94 blood samples were collected from buffalo in slaughter house in al Al-Najaf city by using anticoagulants tubes, from a period extended from August, 2017 into December 2017, the samples including different ages and both sex of buffalo, then the samples directly transport in ice box into laboratory to performing direct smear examination.

Direct Microscopic examination

Direct microscopic examination of blood samples was done by using Giemsa stain (8) to preparation of thin and thick smears .

Blood DNA Extraction

Genomic DNA from blood samples were extracted by using gSYAN DNA mini kit extraction kit (Frozen Blood) Geneaid. USA, and done according to company instructions. The extraction method was don depend on the manufacturing instructions by blood DNA Protocol extraction method by using (11 mg/ml) proteinase K (9).

Nanodrop

The extracted DNA was estimated by nanodrop device at 260/280nm, and then kept at deep freezer until used in PCR method (9).

Polymerase chain reaction (PCR)

PCR technique was performed for indirect detection blood borne *Babesia* sp protozoa from blood of buffalo, the method was carried out according to method described by (9), the PCR primers that used in this study were design in this study by using NCBI Genbank data base and primer 3 plus (10), and provided by Macrogen company, Korea as in table 1.

Table 1: PCR primers with their nucleotide sequence and amplicon size

Primers	Sequence	Amplicon
Babesia sp.	F GGCCGTTCTTAGTTGGTGGA R TGTGTACAAAGGGCAGGGAC	357 bp

NCBI-Genbank: *Babesia* sp. (KF928959.1).

PCR master mix preparation

PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions (Table 2).

Table 2: PCR Master mix preparation

PCR Master mix	Volume
DNA template	5µl
Forward primer (10 pmol)	1.5µl
Reveres primer (10 pmol)	1.5µl
PCR water	12µl
Total volume	20µl

After that, the PCR mix that revealed in table above placed in AccuPower PCR -PreMix that contain all other PCR components which needed to reaction such as (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes transferred into vortex centrifuge for 3 minutes. Then transferred into thermocycler.

PCR Thermocycler Conditions

PCR thermocycler conditions were done by using convential PCR thermocycler system (Table 3).

Table 3: PCR Thermocycler Conditions

PCR step	Temperature	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	
Annealing	58C	30sec	30 cycle
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

PCR product analysis

The PCR products (503bp) were examined by electrophoresis in a 1% agarose gel using 1X TBE buffer, stained with ethidium bromide, and investigation under UV transilluminator.

DNA sequencing method

DNA sequencing method was performed for species typing of positive *Babesia* sp. isolates by PCR technique. The genetic analysis done by phylogenetic tree analysis between local species isolates and NCBI-Blast submission local species. Then the identification species isolates were submitted into of NCBI-GenBank. The PCR products were sent to Macrogen Company in Korea in ice bag by DHL for performed the DNA sequencing by AB DNA sequencing system. The DNA sequencing analysis was conducted by using Molecular Evolutionary Genetics Analysis version 6.0. and multiple sequence alignment analysis of the partial ribosomal rRNA gene based ClustalW alignment analysis and the evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method.

Results

Blood Smear

The microscopic examination includes direct identification of *Babesia* spp. from blood samples of buffalo by using Giemsa stain blood smear method. This method includes identification based on morphological characterization of haemoprotozoa, where, the *Babesia* sp. characterized by presence merozoites infective stage pear-like shaped which found inside RBCs and 1-1.5 µm in long and 0.5- 1.0µm in wide and seen as pairs with obtuse angle, as show in figure 1.

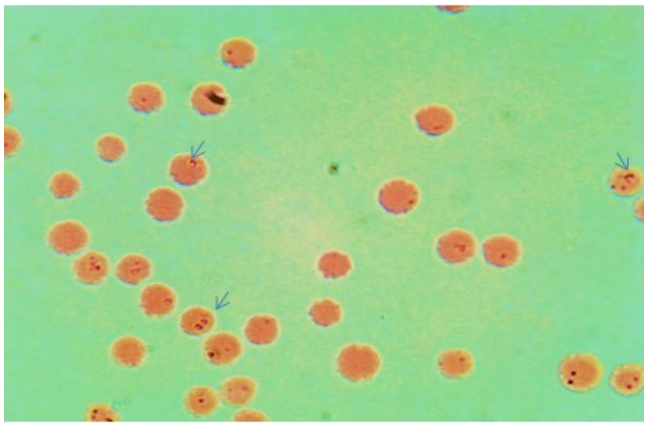

Figure 1: Thin blood smear microscopic image showing presence merozoites infective stage pear-like shaped for *Babesia* which inside RBCs of buffalo. Blood samples. Giemsa stain (100x).

Prevalence of *Babesia* sp

Prevalence of *Babesia* sp. in blood samples of buffalo were studied according to limited period of time include only three months extend from August into September 2017, and according to animals' gender, as well as ages of animals. The study included collection of a 94 blood samples from buffalo in slaughter house in al Al-Najaf city. The *Babesia* sp. were show high prevalence at 45.74%. The present study includes 69 male and 25female buffalo blood samples and the prevalence and statistical analysis were show in table 4.

Table 4: Prevalence of *Babesia* sp. related to sex

Gender	No. of tested samples	No. of positive *Babesia* sp.	Percent %
Male	69	30	43.48
Female	25	13	52.00
Total	94	43	45.74

Chi-square value (x^2: 0.537) not significant at p value = 0.463.

The *Babesia* sp. Prevalence and statistical analysis related to animals ages were show in table 5. The *Babesia* sp. Prevalence and statistical analysis related to study periods were show in table 6.

Table 5: Prevalence of *Babesia* sp. related to age

Age	No. of tested samples	No. of positive *Babesia* sp	Percent %
Young (1month to 6month)	16	2	12.50
Adult (6month-2years)	28	26	92.86
Old (over 2 years)	50	15	30.00
Total	94	43	45.74

Chi-square value (x^2: 19.631) significant at p value = 0.0001.

Table 6: Prevalence of *Babesia* sp. related to month of study

Periods	No. of tested samples	No. of positive *Babesia* sp	Percent %
August	14	4	28.57
September	24	15	62.50
October	56	24	42.86
Total	94	43	45.74

Chi-square value (x^2: 4.567) Non-Significant at p value: 0.102.

Molecular Study

The molecular study results were included PCR technique for confirmative detection and DNA sequencing methods for *Babesia* species typing.

Polymerase Chain Reaction (PCR)

The PCR technique was used for specific in direct confirmative detection of *Babesia* sp. from buffalo that only show positive in direct smear of microscopic examination method. This technique was depending on primers design of small subunit ribosomal genes in these parasites. The PCR results were show high specific and accurate confirmative detection which appeared at 357bp on 1% agarose gel electrophoresis (Figure 2).

DNA Sequencing analysis

DNA sequencing analysis results were including *Babesia* species typing based on phylogenetic tree analysis and ClustalW alignment analysis by using (MEGA 6.0) between local species isolates and NCBI-Blast species recorded isolates. Then confirmative by NCBI-BLAST Homology Sequence Identity. After that identified species isolates were deposited into of NCBI-GenBank to get Genbank accession number for each *Babesia* species isolates (Figure 3) (Table 7 and 8).

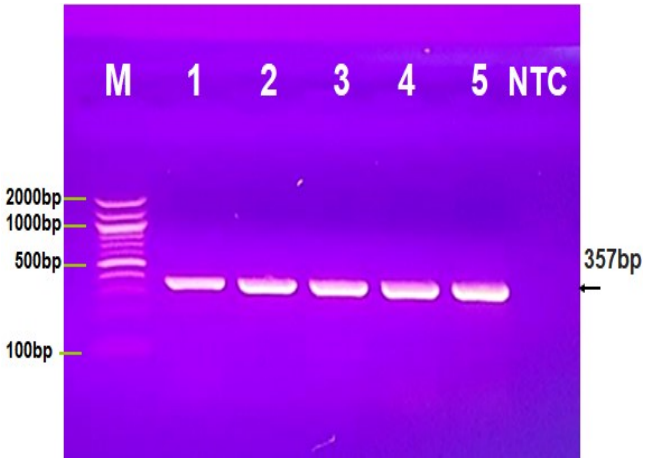

Figure 2: Electrophoresis of PCR reaction results for 18S_ribosomal RNA gene *Babesia* sp. of buffalo blood samples, using 1% agarose gel and DNA marker ladder (2000-100bp), Lane (1-4) positive PCR *Babesia* sp. from buffalo blood samples at 357bp PCR product size and lane (NTC) Non template negative control.

Table 7: The total prevalence of identified *Babesia* species isolates based on DNA sequencing analysis

Babesia sp	No. of tested samples	No. of positive samples	Percent %
Babesia bovis	94	36	38.30
Babesia bigemina	94	7	7.45
Total	94	43	45.74

Chi-square value (x^2: 25.35) Significant at p value: 0.0.

Discussion

The study performed in three months of summer season (August into September 2017) due to high distribution of transmitted vectors in this time of year. This method includes identification of haemoprotozoa based on morphological characterization when used in thin and thick blood smears method and it has been considered to be the standard technique for routine diagnosis, because itis still cheap and fast methods (11). The morphologic characteristics observed on microscopic examination of blood smears do not allow differentiation between *Babesia* species so that the molecular identification is the best of choose .

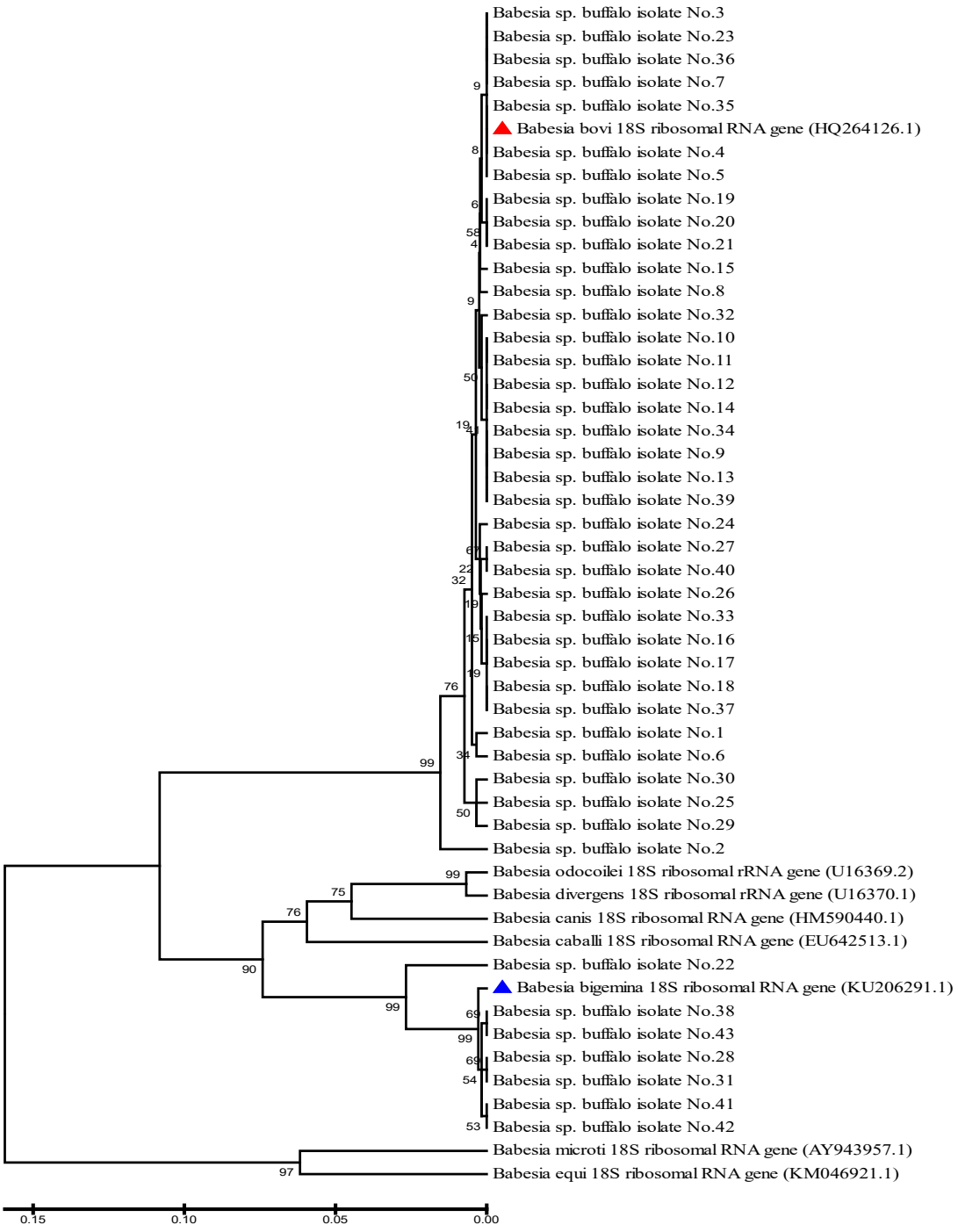

Figure 3: Phylogenetic tree analysis based on the partial sequence of 18S ribosomal rRNA gene in local *Babesia* species buffalo isolates (No.1-No.43) that used for Theileria species genetic identification. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method (MEGA 6.0 version). The local *Babesia* sp. isolates (No.1-No.21, No.23-No.27, No.29-No.30, No.32-No.37, and No.39-No.40) were show closed related to NCBI-Blast *Babesia* bovis (HQ264126.1) and The local *Babesia* sp. isolates (No.22, No.28, No.31, No.38, No.41, No.42, and No.43) were show closed related to NCBI-Blast *Babesia bigemina* (KU206291.1) whereas, other the NCBI-Blast *Babesia* species were show different and out of tree.

Table 8: NCBI-BLAST Homology Sequence Identity between local *Babesia* sp. buffalo isolates and closed related to NCBI-Blast *Babesia* bovis (HQ264126.1) and *Babesia bigemina* (KU206291.1)

Local *Babesia* sp. isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence Identity	
		Identical *Babesia* sp.	Identity (%)
Babesia sp. No.1	MH503811	*Babesia bovis*	99%
Babesia sp. No.2	MH503812	*Babesia bovis*	97%
Babesia sp. No.3	MH503813	*Babesia bovis*	100%
Babesia sp. No.4	MH503814	*Babesia bovis*	100%
Babesia sp. No.5	MH503815	*Babesia bovis*	100%
Babesia sp. No.6	MH503816	*Babesia bovis*	99%
Babesia sp. No.7	MH503817	*Babesia bovis*	100%
Babesia sp. No.8	MH503818	*Babesia bovis*	99%
Babesia sp. No.9	MH503819	*Babesia bovis*	99%
Babesia sp. No.10	MH503820	*Babesia bovis*	99%
Babesia sp. No.11	MH503821	*Babesia bovis*	99%
Babesia sp. No.12	MH503822	*Babesia bovis*	99%
Babesia sp. No.13	MH503823	*Babesia bovis*	99%
Babesia sp. No.14	MH503824	*Babesia bovis*	99%
Babesia sp. No.15	MH503825	*Babesia bovis*	99%
Babesia sp. No.16	MH503826	*Babesia bovis*	99%
Babesia sp. No.17	MH503827	*Babesia bovis*	99%
Babesia sp. No.18	MH503828	*Babesia bovis*	99%
Babesia sp. No.19	MH503829	*Babesia bovis*	99%
Babesia sp. No.20	MH503830	*Babesia bovis*	99%
Babesia sp. No.21	MH503831	*Babesia bovis*	99%
Babesia sp. No.22	MH503832	*Babesia bigemina*	95%
Babesia sp. No.23	MH503833	*Babesia bovis*	99%
Babesia sp. No.24	MH503834	*Babesia bovis*	99%
Babesia sp. No.25	MH503835	*Babesia bovis*	99%
Babesia sp. No.26	MH503836	*Babesia bovis*	99%
Babesia sp. No.27	MH503837	*Babesia bovis*	99%
Babesia sp. No.28	MH503838	*Babesia bigemina*	99%
Babesia sp. No.29	MH503839	*Babesia bovis*	99%
Babesia sp. No.30	MH503840	*Babesia bovis*	99%
Babesia sp. No.31	MH503841	*Babesia bigemina*	99%
Babesia sp. No.32	MH503842	*Babesia bovis*	99%
Babesia sp. No.33	MH503843	*Babesia bovis*	99%
Babesia sp. No.34	MH503844	*Babesia bovis*	99%
Babesia sp. No.35	MH503845	*Babesia bovis*	100%
Babesia sp. No.36	MH503846	*Babesia bovis*	100%
Babesia sp. No.37	MH503847	*Babesia bovis*	99%
Babesia sp. No.38	MH503848	*Babesia bigemina*	99%
Babesia sp. No.39	MH503849	*Babesia bovis*	99%
Babesia sp. No.40	MH503850	*Babesia bovis*	99%
Babesia sp. No.41	MH503851	*Babesia bigemina*	99%
Babesia sp. No.42	MH503852	*Babesia bigemina*	99%
Babesia sp. No.43	MH503853	*Babesia bigemina*	99%

The present study was showed prevalence of haemoprotozoa at (45.74%). Study in Egypt byHazem *et al.* (12). Their results are agreement with our observation, who recorded a higher prevalence of Babesiosis in buffaloes than in cattle by blood films examination and revealed that, the infection rate with Babesiosis was 22.47% of cattle and 51.28% of buffaloes. The presence of ticks or history of tick infestation to the animal was associated with the presence of babesiosis and the high incidence of babesiosis in domestic cattle and buffalos, it is possibly due to tick

infestation, was more distribution in buffaloes during summer season lead to risk factors associated with higher prevalence of Babesiosis (13). The present study was show the high prevalence of *Babesia* sp. in female at (52.%) than male was 43.48% but non-significant differences at p value: 0.463. this prevalence was agreement with the report of Khattak *et al.* (14) who observed females buffalo more susceptible to *Babesia* sp. at 34.06 % than males at 22.70%. Overall prevalence of haemoprotozoan parasite in female buffalo was higher than male, this higher prevalence in female population may be due to hormonal disturbances which pretense it to weakened immune system. Our results were prevalence of *Babesia* sp. was 12.50%, 92.86% and 30% in young, adult and old age respectively and with significant differences at p value: 0.0001. This present prevalence was supports by Hazem *et al.* (12), who revealed that, adult animals 60% were more infected by Babesiosis as compared with calves 40%. The increase of *Babesia* infection with increasing animal age, was mainly due to the postponed infection caused by restriction of calf movement by keeping them indoors of farmers (15).

The present study was performed in this time of year, because most of the animals exposed to haemoprotozoa during summer months may be due to high abundance of vectors in these seasons of the year (16,17). The prevalence of *Babesia* sp. (28.57%), (62.50%) and (42.86) in August, September and October respectively and with non-significant differences at p value: (0.102). Our finding was agreement with Maharana (18) who revered that risk of babesiosis was significantly higher in summer season followed by rainy compared to winter season and reported high prevalence babesiosis in buffaloes. The higher infection of *Babesia* sp. in summer season may be explained by the highest abundance of the ticks in these months and this observation agreed with that previously reported by Alim *et al.* (19).

The present study was identified two *Babesia* species, *Babesia* bovis and *Babesia bigemina* based on phylogenetic tree analysis and homology sequence identity from local *Babesia* sp. isolates. Our results were show high prevalence of *Babesia bovis* 38.30% were closed related to NCBI-Blast *Babesia* bovis (HQ264126.1) with homology sequence identity 97-100% followed *Babesia bigemina* 7.45% were closed related to NCBI-Blast *Babesia bigemina* (KU206291.1) with homology sequence identity 95-99% as show in DNA sequencing analysis, with significant differences at p value: (0.0). less studies about the molecular identification of *Babesia bigemina* and *Babesia* bovis in water buffalos in Iraq, but recent study in Wasit province, of Iraq was consistence with our finding by Alkefari *et al.* (20), who recorded the incidence of *Babesia bigemina* in apparently healthy buffaloes using of three different diagnostic assays and their results show revealed 11.73% positive animals by PCR. Another study was agreement with our results by Aaiz and Sabbar (21), who recorded high prevalence of *Babesia bovis* 47.91% in alive and slaughtered cattle from different areas and abattoir of Al-Qadisiyah province of Iraq. The high prevalence of *Babesia* bovis in buffalo of our study may be due to most buffalo were appear to be bearing the infection predominantly as a carrier host.

Worldwide distribution prevalence of *Babesia bigemina* and *Babesia* bovis in cattle and buffalo were show disagreement with our finding, study in the northeastern region of Thailand, by Terkawi *et al.* (22) who reveal that PCR prevalence of *B. bovis* and *B. bigemina* was 11.2% and 3.6% respectively. Study in Egypt by Ibrahim *et al.* (23) who recorded prevalence of *Babesia bigemina* and *Babesia* bovis in cattle and water buffalos by Molecular and serological methods were 10.42% and 4.17% by PCR and 15.63% and 11.46% by ELISA, respectively. Another Study in Vietnam by Yan *et al.* (24) who recorded prevalence of *Babesia bigemina* and *Babesia* bovis in cattle and water buffalos by Molecular and serological methods were 23.3% and 0% by PCR, 37.2% and 9.3% by ELISA and 27.9% and 18.6% by IFAT, respectively.

In conclusion, this study concluded that Phylogenetic tree and homology sequences identity was show accurate in differentiation of *Babesia* species and these species can be isolated at high prevalence from local water buffalo from slaughter house in Al-Najaf city, of Iraq.

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