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MOLECULAR DIAGNOSIS OF *BABESIA SPECIES* IN LOCAL SHEEP IN MOSUL CITY/IRAQ

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ABSTRACT

The present research was conducted to distinguish *Babesia spp* in sheep in different locations in the city of Mosul - Iraq, using conventional molecular methods. One hundred and eighty blood samples were collected from local sheep of different age groups and of both sexes for the period from 1/12/2023 to 1/5/2024. The blood samples were examined using PCR technique, genetic sequencing, and a phylogenetic tree based on the 18srRNA gene. Our results showed that several positive samples infected with Babesia parasite reached 132 samples, at a rate of 73.3%, with a reaction result of 300 base pairs. In this research, three new isolates of the *Babesia ovis* (LC799506.1, LC799507.1, and LC799508.1) and three new isolates of the *Babesia motasi* (LC799509.1, LC799510.1, and LC799511.1) were recorded in sheep in Mosul city. The *Babesia ovis* isolates fall within the scope of the genetic sequence and phylogenetic tree within the same clade and that these isolates were closely related to both the Turkish (HM241889.1) and German (AY260178.1) isolates. As for the three isolates of *Babesia motasi*, there was closeness between the two isolates LC799510.1 and LC799511.1 Also, these two isolates were close to the Chinese isolates (MH899762.1 and (MF361088.1) and the British isolate (KU2345270.1). While the isolate LC799509.1 was close to the Iraqi isolate recorded in Diwaniyah KP998110.1), as well as to the isolate recorded in Pakistan (KY765559.1). In conclusion, Veterinarians and researchers should consider that *Babesia ovis* and *Babesia motasi* were diagnosed in sheep for the first time in Mosul city \ Iraq.

Key word: *Babesia genus*, *Babesia ovis*, *Babesia motasi*, molecular diagnosis

INTRODUCTION

Sheep are an important source of livestock in the Arab world as well as in many countries of the world (Sulaiman *et al*., 2022 Mahmood and Ahmed, 2022), Sheep of Mosul related to Asian fat-tailed sheep and there are four local breeds, they are Awassi, Naimi, Arabi, and Karadi (Alkass *et al*., 2023). Awassi sheep comprise 60% of the total local sheep. Sheep of all breeds are an important source of meat, milk and wool production (Ali *et al*., 2022, Salih and Ahmed, 2022). The *Babesia* parasite lives obligatory inside red blood cells of hosts and is transmitted by hard ticks and this parasite belongs to the phylum Apicomplexa (Lee *et al*., 2018). Domestic and wild animals are infected with more than 100 *Babesia* spp. (Aktas, 2007). In sheep, there are three species of *Babesia* mentioned causing babesiosis: *Babesia ovis*, *Babesia motasi*, and *Babesia crassa*. *Babesia ovis* is highly pathogenic

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species in sheep and goats with mortality rates of 30-50%, while *Babesia motasi*, and *Babesia crassa* are non-pathogenic or less pathogenic (Mghirbi *et al*., 2013).

Diagnosis of *Babesia* by examining blood smear stained with Giemsa (Hatem, 2020). Sometimes there may be difficulty in diagnosis of parasite in animals with very low parasitemia and even in some acute cases. Moreover, distinguishing the species of *Babesia* by relying only on microscopic examination of thin blood smears is difficult, especially for veterinarians with little experience, because most *Babesia* species have the same morphological characteristics (Hasheminasab *et al*., 2018). Therefore, microscopic examination needs support and confirmation. So molecular techniques based on DNA, including polymerase chain reaction (PCR) are used for diagnosis of *Babesia spp*. with a high degree of sensitivity (Naderi *et al*., 2017).

There are some studies using molecular detection of different *Babesia* species infecting sheep conducted in different countries (Jefferies *et al*., 2003 and Altay *et al*., 2008). For example, a high incidence of *Babesia ovis* infection has been recorded in Nigeria 36.9% using PCR technology (Adewumi *et al*., 2022). While in north of Iran the prevalence of *Babesia* was 5% (Haghi *et al*., 2013). In Pakistan, the reported percentage of infection *with B. ovis* using PCR were 34% (Kumar *et al*., 2018) and 50% (Iqbal *et al*., 2011). The percentage of infected sheep *with B. ovis* using PCR were 8.25% (Aktas, 2007) and 16.27% (Altay *et al.* 2008) in Turkey and 17.4% in in Tunisia (Rjeibi *et al.* 2014).

Given that studies on the *Babesia* genus and its species in Mosul city, especially in sheep, appear to be limited, this work was conducted in order to determine Babesia infection on a molecular basis and determine their genetic diversity (phylogenetic tree).

MATERIALS AND METHODS

Ethical approval

The ethics of this research were previously approved by the institution Animal Care College of Veterinary Medicine\University of Mosul with an authorized ID of UM. Vet. 2023.104

Blood samples collection:

A number of ٨٠ blood samples were randomly collected from male and female sheep of different age, including healthy animals and animals suffering from distinctive symptoms of babesiosis from different localities of Mosul city.

Blood samples were taken from the jugular vein after sterilizing the area with 70% ethyl alcohol using sterile syringes. The blood samples were kept in EDTA tubes, after that, the samples were transferred to the Parasitology Laboratory (Al-Obaidi and Alsaad, 2004).

Molecular diagnosis of *Babesia spp*.

DNA extraction from blood

DNA was extracted from sheep blood samples (180 samples) using a ready-made extraction kit (Qiagen). The extracted DNA was preserved in a freezer until use. The PCR reaction was performed using the primer shown in Table (1).

Table 1: The primers which were used for diagnosis *Babesia genus.*

Primer	Sequence	Annealing
18s rRNA-F	GGTAATTCCAGCTC CAATAG	58
18s rRNA-R	ACCACCAAAATAG AACCAAAGTC	

The reaction tubes were loaded with 1ml of each primer, 4ml of the isolated DNA, 10ml of master mix biolaps and 4ml of PCR grade water. The reaction tubes were placed in the Thermocycler device to complete the polymerization reaction according to the reaction program in Table (2).

Table 2: Cycling condition for amplification of *Babesia* gene

Steps	Temperature	Time	Cycle number
Initial denaturation	95	6 min.	1
Denaturation	95	45 sec.	35
Annealing	58	1.0 min.	
Extension	72	1.0 min.	
Final extension	72	5 min.	1

A 4µl of each PCR product was loaded into the hole of agarose gel 2% and the 100 bp DNA marker (Biolaps), 4µl, was used as a standard molecular marker. The electrophoresis process of the PCR reaction product was carried out at 60 V for 60-70 min. The gel was removed and immersed in a container containing distilled water with a few drops of safe red stain for 30 minutes. the gel was removed from the dye solution and placed in UV-Transilluminator (Gel Documentation). The amplification product bands were photographed using a digital camera.

DNA sequencing

The 20 PCR-positive samples were sent to the Genetic Analyzer 3130 (Hitachi, Japan) and matched with NCBI according to the BLAST program for the purpose of studying the genetic sequence of nitrogenous bases and determining the type of *Babesia* with a study of the phylogenetic tree of the *Babesia species* diagnosed based on the Mega 11 program.

RESULT

Identification of the *Babesia* species based on conventional PCR technology

The results of using the NanoDrop device showed that the concentration of DNA extracted from 180 sheep blood samples ranged from 39.2-345 nanogram/microliter, and its purity ranged between 1.7 -1.9. All the DNA samples extracted from the sheep's blood were loaded onto the agarose film at a concentration 1%. Most of the DNA bands were clearly observed, as follows (Figure 1).

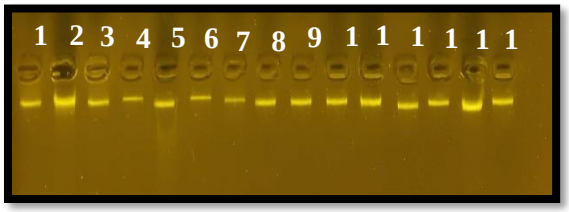

Figure (1): Showing the DNA bands extracted from sheep blood in the city of Mosul

Genetic sequencing results:

180 blood samples of sheep were randomly selected and examined with the PCR technique. The number of blood samples in which the *Babesia* species was diagnosed by conventional PCR technique was 132 with a percentage of 73.3% (Table 3).

Table 3: Showing the percentage of *Babesia* diagnosis in 180 blood samples based on the conventional PCR technique.

Diagnostic technique used	Number of positive samples	Percentage of infection
Conventional polymerase chain reaction	132	73.3

The results of the study also showed, based on conventional PCR technology, the diagnosis of the genus *Babesia* for the first time in Mosul city in 132 blood samples from sheep. The replication results appeared in the form of bands on agarose gel at a concentration of 2% and the molecular weight of the band was 300 a base pair (Fig 2).

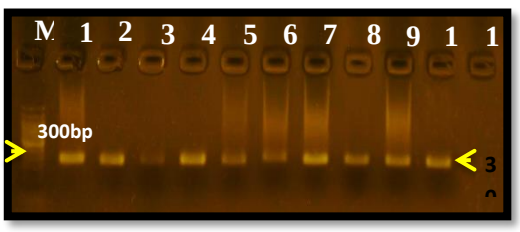

Fig. (2): The results of the PCR reaction for the *Babesia* genus, for the 18srRNA region, and the reaction product of 300 bp.

a total of 20 samples had a positive PCR result sent to Psomagena USA center that

were diagnosed in the city of Mosul, including obtaining a sequence number for the local genetic sequences of the *Babesis spp*. (MN493171.1 and MN493169.1) and the local genetic sequences of the *Babesia ovis* (LC 714840.1, ON307468.1 ON 307467.1, ON307466.1). The results of the genetic sequencing of the 18SrRNA gene of the *Babesia* parasite showed the presence of a genetic sequence for 18 samples. The local genetic sequence of the *Babesia ovis* isolate isol 3 was the most frequent, as shown in Table (4) and Fig. (3).

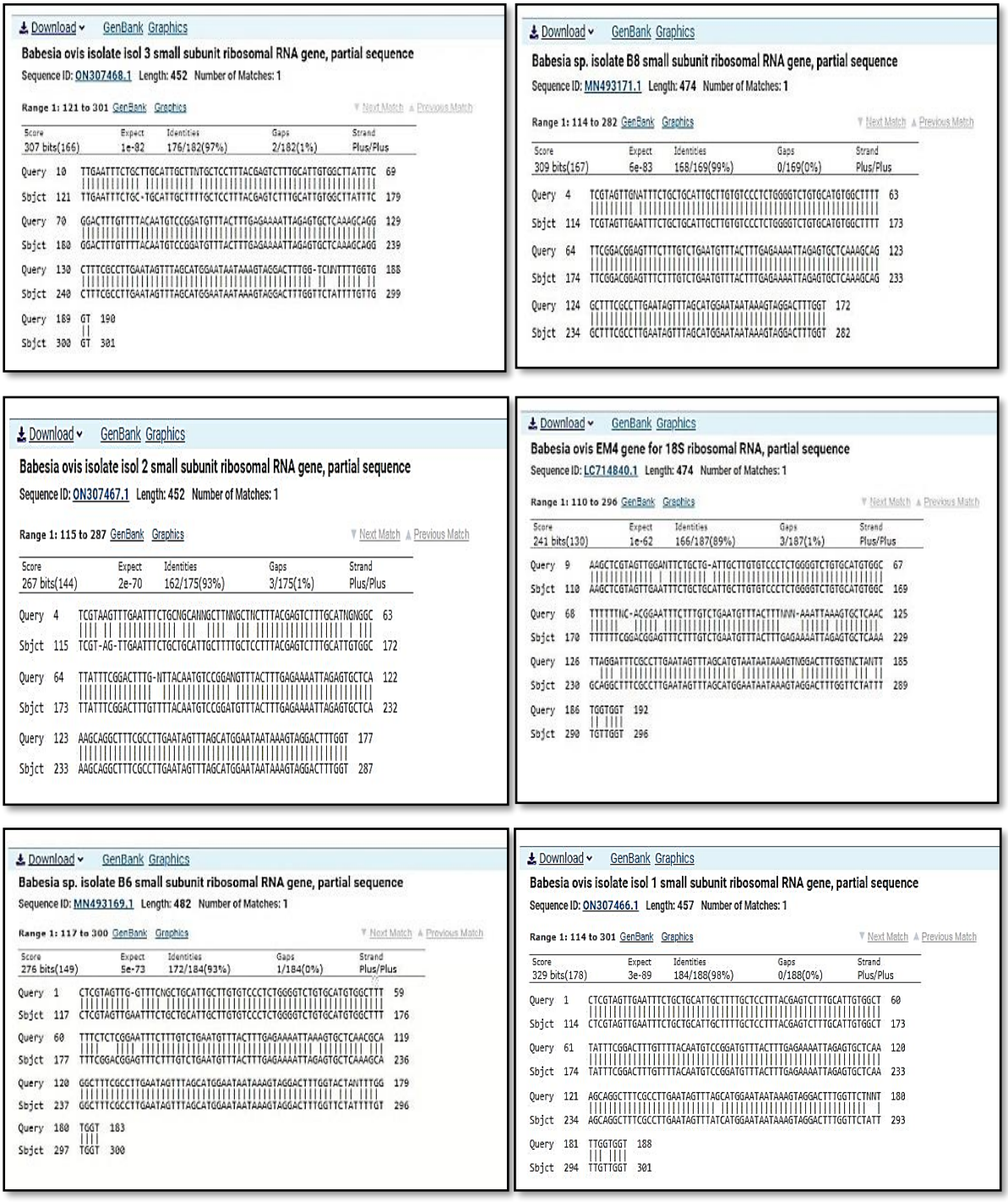

Fig. (3): Taxonomic symbol of strains of *Babesia* in sheep in Mosul city

Table (4): Local genetic sequences of the *Babesia* diagnosed in 20 positive reaction products of PCR technology

Name of isolate	Number of nucleotides	Genebank registration number	Number of isolation repetitions
Babesia sp. Isolate B8	474	MN493171.1	1
Babesia ovis EM4 gene	474	LC 714840.1	1
Babesia ovis isolate isol 3	452	ON307468.1	9
Babesia ovis isolate isol 2	452	ON 307467.1	3
Babesia ovis isolate isol 1	457	ON307466.1	2
Babesia sp. Isolate B6	482	MN493169.1	2

Three new strains of the *Babesia ovis*, and three new strains of the *Babesia motasi*, were recorded, and they are, as shown in Table (5).

Table 5: *Babesia* isolates from sheep blood samples diagnosed by (NCBI) according to the BLAST program

The No. of *Babesia* isolate in NCBI	Name of isolate
LC799506.1	*B. ovis* BEBA1
LC799507.1	*B. ovis* BEBA2
LC799508.1	*B. ovis* BEBA3
LC799509.1	*B. motasi* BEBA4
LC799510.1	*B. motasi* BEBA5
LC799511.1	*B. motas*i BEBA6

Result of phylogenetic analysis

In this study, three new local isolates of the *Babesia ovis* were obtained in sheep in the city of Mosul, and when comparing the extent of proximity and divergence of these isolates with the isolates registered in the gene bank, it was revealed that five clades were obtained and that our isolates in this study (LC799507.1 *Babesia ovis* BEBA2, LC799506.1 *Babesia ovis* BEBA1 and LC799508.1 *Babesia ovis* BEBA3) formed one clade close to each other by 11%. Also, in the same clade, there was a similarity with the isolates recorded in Turkey (HM241889.1 *Babesia ovis* Turkey) with a percentage was 11%, and the isolate recorded in Germany (AY260178.*1 Babesia ovis* Germany), with a percentage of 27% as shown in the Figure (4).

Also in this study, three isolates of *Babesia motasi* were obtained from sheep in Mosul city, these isolates were used for genetic analysis with various isolates in countries around the world, and three clades were obtained. It was observed that there was affinity between the two ours isolates (LC799510.1 *Babesia motasi* BEBA5 and LC799511.1 *Babesia motasi* BEBA6) with each other at a rate of 23%, these two isolates were also close to the isolates recorded in China and United kingdom (MH899762.1 *Babesia motasi* China, MF361088.1 *Babesia motasi* China, and KU234527.1 *Babesia motasi* United Kindom), respectively, also at a rate of 23%. As for our isolate (LC799509.1 *Babesia motasi* BEBA4), it was close to the isolates recorded in Diwaniyah/Iraq (KP998110.1 *Babesia motasi* Iraq Diwania) and in the Pakistan (KY765559.1 *Babesia motasi* Pakistan) with a percentage 23% as shown in the Figure (5).

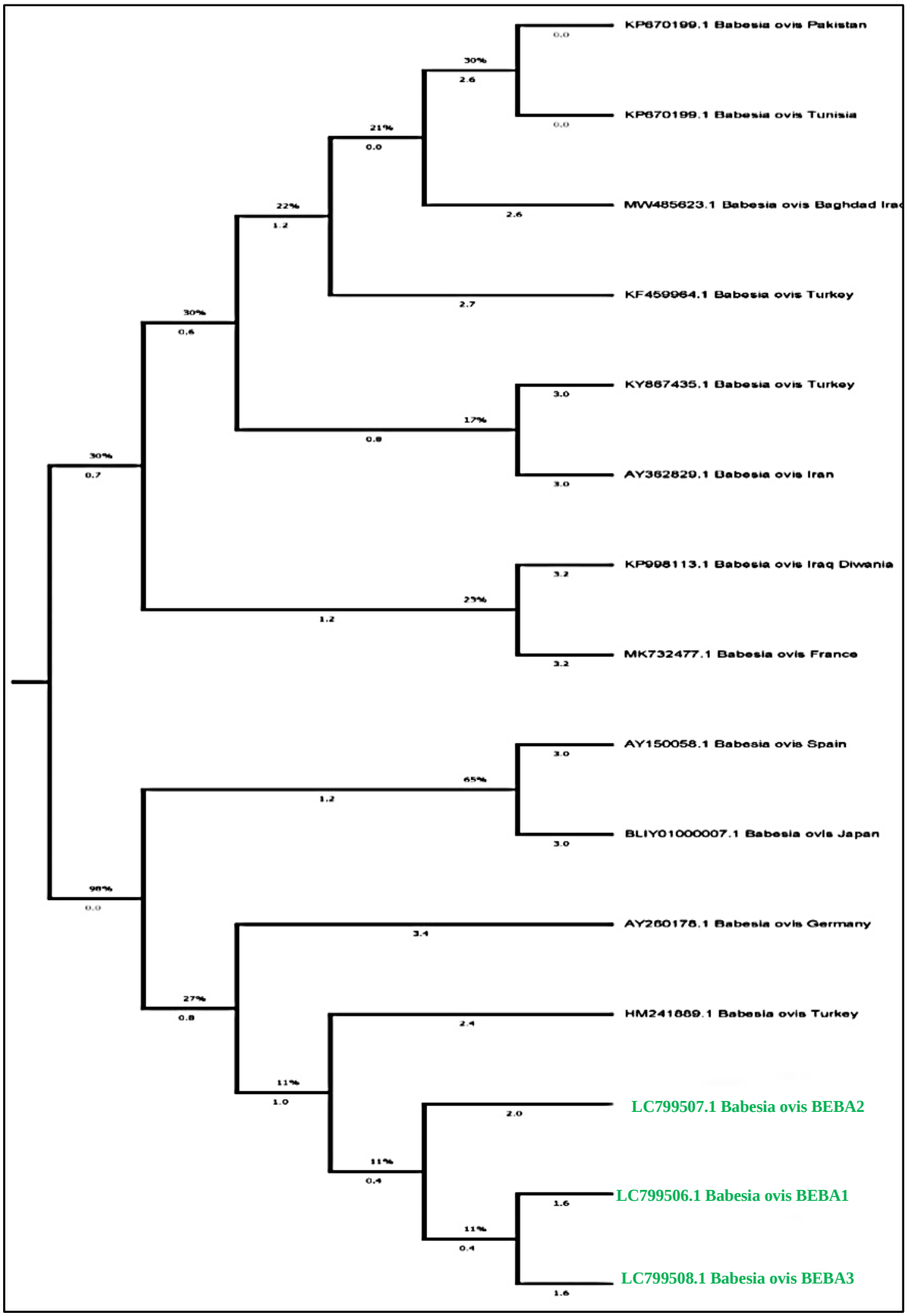

Fig. (4): The phylogenetic tree of different isolates from the Gene bank with ours isolates of *Babesia ovis* in this studv

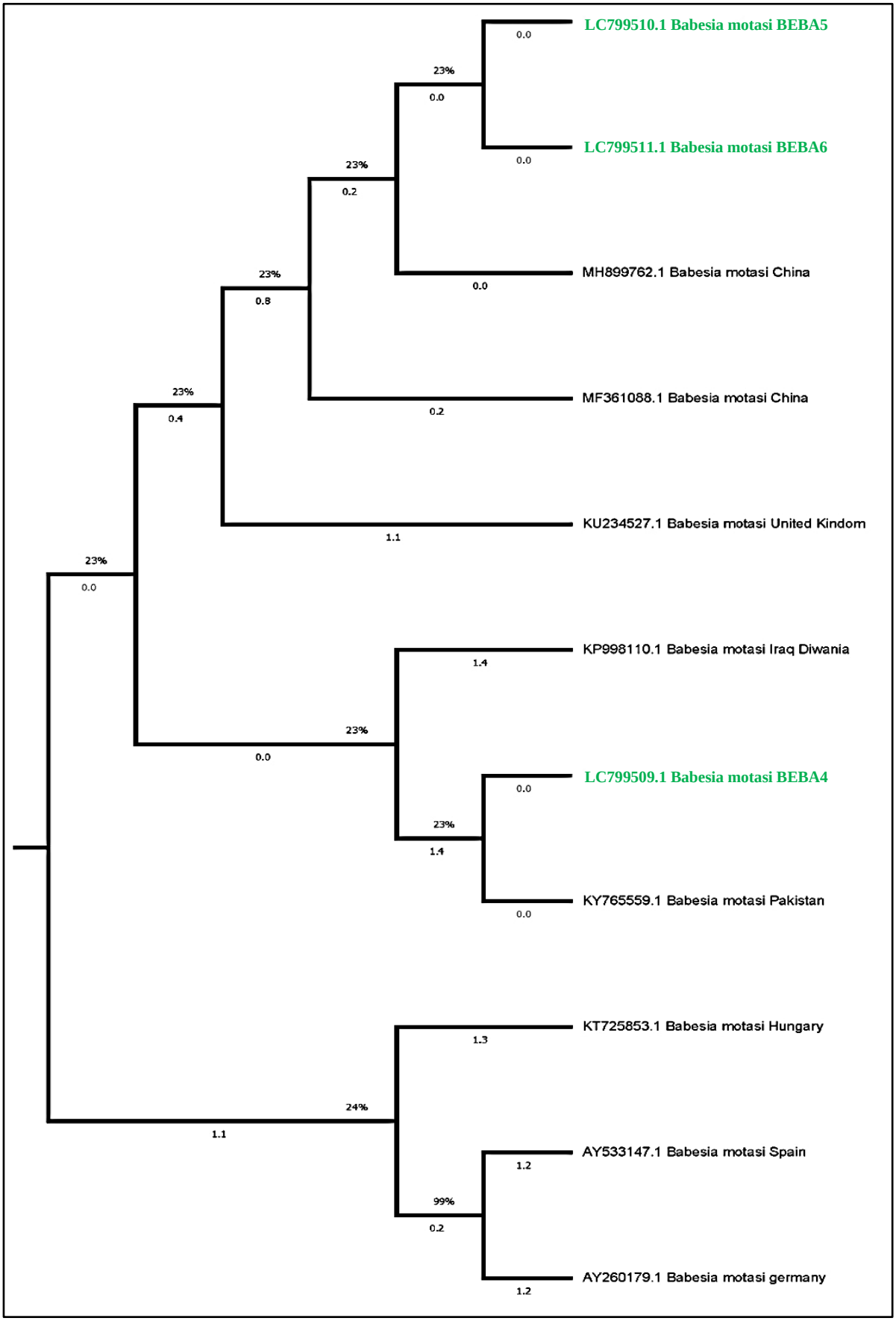

Fig. (5): The phylogenetic tree of different isolates from the Gene bank with ours isolates of *Babesia motasi* in this study

DISCUSSION

Molecular method such as PCR was successfully used in veterinary parasitology in recent years for diagnosis the infection with blood protozoa such as *Babesia species* (Inci *et al*., 2010, Noman 2013, Altay *et al*., 2007b). In this study, the *Babesia species* in sheep were diagnosed and identified for the first time in the city of Mosul by using the conventional PCR technique with a very high percentage, which was 73.3%. *Babesia species* have similar morphological characteristics, and PCR technology allows the identification and diagnosis of this parasite at a low level of parasitism and also in cases of mixed infection in animals (Aydin *et al*., 2013). The result of this study is close to a previous molecular survey study on the *Babesia spp.* in cattle and goats, as it reached 73.55% (Mohanta *et al.*, 2023). Our results are higher than the recorded infection rate 5% with *Babesia* in sheep in Iran (Haghi *et al.*, 2013). Through studying the genetic sequence of the *Babesia* parasite in sheep in Mosul city, it was found that most of the isolates belonged to the species *Babesia ovis* and that the isolate *Babesia ovis* isolate isol 3 was the most frequent among the isolates. In addition, three new local isolates of the *Babesia ovi*s in Mosul city were recorded.

The *Babesia ovis* was diagnosed in blood smears and by PCR in sheep and goats in Erbil province- Iraq, and the incidence of *Babesia ovis* in sheep was higher 20.41% than in goats 9.04% (Hassan, 2020). *Babesia ovis* has the ability to cause the disease and the appearance of distinct clinical symptoms in sheep more than in goats (Bilgic *et al*. 2017).

The results of this study agreed with previous studies in Tunisian sheep, where the molecular prevalence of *Babesia ovis* was highly significant at a rate of 17.4% (Rjeibi *et al*., 2014) and 7.8% (Rjeibi *et al*., 2016). Also, Iqbal *et al*. (2011) reported higher prevalence (50%) of *B.ovis* in Pakistan. Mohanta *et al*. (2023) in his molecular survey study, indicated a high incidence of *Babesia ovis* in goats in Bangladesh, at a rate of 32.26%. In Turkey, *Babesia ovis* species were diagnosed in sheep and goats as well, using molecular techniques, at a rate of 5.43 (Aydin *et al*., 2013), 2.7% (Altay *et al*., 2007b). In another study in Baneh, Iran, the *Babesia ovis* was also diagnosed in 66 blood samples from sheep, at a rate of 86.4%, based on PCR methods (Gh *et al*., 2020). The results of microscopic and sequencing analysis showed a high incidence *of Babesia ovis* in sheep in Nigeria was 61.1%, 38.9%, respectively (Adewumi *et al*., 2022). There were also similar results it has been recorded in Turkey and Iran where goats and sheep are found infected by only *B. ovis* is (Altay *et al*., 2007b; Esmaeilnejad *et al.*, 2014). In Shayan's study (2008) on 20 infected sheep, although the morphological and biomert-rical parameters were similar to the species of *Babesia motasi*, the results of the molecular examination using the 18SrRNA primer confirmed that it was *Babesia ovis* and not *Babesia motasi*.

In this study, the species *Babesia motasi* was diagnosed, and three new local isolates were also identified. This result agreed with Haghi *et al*. (2013) who diagnosed both species (*B.ovis and B.motasi*) in sheep and goats in north of Iran. In Spain, *B. ovis* and *B. motasi* are occurred in sheep have a total infection rate of 2.5% and 2%, respectively, Nagor *et al*. (2004). Uilenberg (2006) referred that *B. ovis* is the main type of genus *Babesia*.

Ours results of the genetic sequence and phylogenetic tree of the *Babesia* parasite in sheep in the city of Mosul indicated that three new isolates of the *Babesia ovis* and three isolates of *Babesia motasi* were recorded, and that the isolates of the *Babesia ovis* fell within the same clade and that these isolates were closely related to each of the Turkish and German isolates, the three isolates of the *Babesia motasi*, closeness was observed between the two

isolates (LC799510.*1 Babesia motasi* BEBA5 and LC799511.*1 Babesia motasi* BEBA6), also, these two isolates were close to the Chinese isolate and the United Kingdom isolate. As for the isolate LC799509.1 *Babesia motasi* BEBA4, it was close to the Iraqi isolate recorded in Diwaniyah (KP998110.1 *Babesia motasi* Iraq Diwania), as well as to the isolate recorded in Pakistan (KY765559.1 *Babesia motasi* Pakistan). Wang *et al*. (2019) referred the *Babesia ovis* is distant from *Babesia motasi, Babesia crassa, and Babesia sp*.

The study of AL-Khaled and Abdul-Hassan, 2015) on the phylogenetic tree of *Babesia spp.* isolated from small ruminants in central Iraq regions showed that *Babesia motasi* No.1 (goat-Iraq) (KP998113.1), *Babesia motasi* NO.2 (sheep-Iraq) (KR264956.1) showed close relationship with the strain of *Babesia motasi (*AY260179) isolated from Netherlands sheep while *Babesia motasi* No.3 (sheep-Iraq) (KR264957.1) appeared high homology and identity with *Babesia motasi* strain isolated from (sheep-Spain) (AY533147.1), while (Rjeibi *et al.*, 2016) indicated that all of the *B.ovis* isolates were within a single clade. Wang *et al*. (2019) confirmed that *Babesia spp*. and *B. motasi* infected the sheep and goat in China.

Hassan (2020) study on the genetic sequence and genetic analysis of the *Babesia ovis* indicated that the *Babesia ovis* diagnosed in Erbil does not differ from all *Babesia ovis* diagnosed in sheep in nearby geographical areas. Abdullah and Ali (2022) reported in their study on the *Babesia* in native sheep in Sulaimani Governorate/ Northern Iraq that *B. ovis* and *B. motasi*, with a high homology degree of nucleotide identity with other nucleotide sequences of *Babesia* spp. in GenBank.

Adewumi *et al*. (2022) indicated that the genetic analysis of *the Babesia ovis* in sheep in Nigeria showed a degree of similarity between the isolates diagnosed in his study and these isolates had a similarity of 100% to the isolates of the Kingdom of Saudi Arabia, and in a ratio of 91-95.55% to each of the Iraqi, French, Turkish, Albanian, Romanian, Italian and Egyptian isolates. In Tunisia, Rjeibi *et al*. (2016) indicated that the *Babesia ovis* isolate had 99.4% similarity to all recently recorded *Babesia ovis* isolates in small ruminants in Tunisia, and also had a percentage 99.4%, 99.2% and 99% of similarity to *Babesia ovis* in Spain (AY150058), Turkey (AY260178), and Iraq (KC778787).

Conclusion

In conclusion, Veterinarians and researchers should consider that *Babesia ovis* and *Babesia motasi* were diagnosed in sheep for the first time in Mosul city \ Iraq.

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التشخيص الجزيئي لأنواع الكمثريات (البابسية) في الأغنام المحلية في مدينة الموصل /العراق

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اجريت الدراسة الحالية للكشف عن طفيلي الكمثريات في الاغنام في مناطق مختلفة من مدينة الموصل – العراق باستخدام الطرق الجزيئية التقليدية والحديثة, جمعت ١٨٠ عينة دم من الاغنام المحلية من فئات عمرية مختلفة ومن كلا الجنسين للفترة من٢٠٢٣/١٢/١ ولغاية ٢٠٢٤/٥/١. فحصت هذه العينات باستخدام تقنية تفاعل البلمرة المتسلسل واجراء التسلسل الجيني وشجرة النشوء الوراثي وذلك بالاعتماد على الجين الحمض النووي الرايبي الريباسيs١٨.

اشارت نتائج الدراسة الحالية ان عدد العينات الموجبة بطفيلي الكمثريات بلغت ١٣٢ عينة وبنسبة ٧٣,٣٪ وبنتيجة تفاعل البلمرة المتسلسل بلغت ٣٠٠ زوجا قاعديا .

اشارت نتائج التسلسل الوراثي وشجرة النشوء والتطور لطفيلي الكمثريات في الأغنام المحلية في مدينة الموصل إلى تسجيل ثلاث عزلات جديدة من طفيلي البابسية الغنمية (LC799506.1 و LC799507.1 و LC799508.1) وثلاث عزلات من طفيلي البابسية موتاسي (LC799509.1 و LC799510.1 و LC799511.1)، وأن عزلات البابسية الغنمية تقع ضمن نطاق التسلسل الجيني وشجرة النشوء والتطور ضمن نفس الفرع الحيوي وأن هذه العزلات كانت مرتبطة ارتباطاً وثيقاً بكل من العزلات التركية والألمانية، امالعزلات الثلاثة للبابسية موتاسي فلقد كان هناك تقارب بين العزلتين LC799510.1 و LC799511. 1 كما أن هاتين العزلتين كانتا قريبتين من العزلة الصينية والعزلة البريطانية. اما العزلة LC799509.1 فقد كانت قريبة من العزلة العراقية المسجلة في الديوانيةKP998110.1 كذلك من العزلة المسجلة في باكستانKY765559.1. وفي الختام، يجب على الأطباء البيطريين والباحثين الأخذ بعين الاعتبار أن بابيزيا أوفيس وبابيزيا موتاسي تم تشخيصهما في الأغنام لأول مرة في مدينة الموصل / العراق.

الكلمات المفتاحية: جنس البابسية ، البابسية الغنمية، البابسية موتاسي، التشخيص الجزيئي