ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Phylogenetic Study of Babesia spp based on 18s rRNA gene isolated from Sheep and Goats in Middle Region of Iraq

Mansour Jadaan Ali Alkhaled , Noora Salah Abdul-Hassan
Department of microbiology and parasitology. College of veterinary Medicine. Univ. of Al- Qadisiya. Iraq

Manuscript Info

Manuscript History:

Received: 15 July 2015
Final Accepted: 22 August 2015
Published Online: September 2015

Key words:

Babesia, Sheep, Goats , Phylogenetic analysis , Sequencing

Corresponding Author

Mansour Jadaan Ali Alkhaled

Abstract

The present study was conducted during the period from September 2014 until April 2015. 163 blood samples was collected from 95 sheep and 68 goats from different regions in AL-Qadisiya province in Iraq and the study designed to using the molecular techniques such as the conventional PCR and the semi nested PCR also the using of sequencing and phylogenetic analysis to determining the parasite species also to determine the effect of the age and sex in animals infection and this study done for the first time in sheep and goats in the middle region of Iraq.

INTRODUCTION

One of the most important disease in small ruminants is the infection with protozoan parasites specially*Babesia*and*Theileria*(1).*Babesia* has a great economic, veterinary and medical important worldwide. it considered the second most commonly found parasites in the mammals blood after Trypanosomes. the disease also described found in birds. The life cycle of *Babesia* have 2 phase the asexual phase reproduce inside erythrocytes while the sexual phase of the life cycle typically takes in Ixodid ticks which acquire and transmit the parasites during their blood meals (2,3).

The disease may cause a wide range of clinical symptoms in domestic animals and human beings Such as acute febrile reaction, fever, jaundice hemolytic anemia hemoglobinuria (4). So the infection can be fatal if associated with stressful Which lead to high economic losses worldwide (5) . The diagnosis of *Babesia spp*. Performs traditionally using Giemsa staining of suspicious blood smears and the morphology of the parasite that have some false morphological diagnosis and in some animal it difficult or impossible due to carriers animal (6). also there are other roles for diagnosis such as polymerase chain reaction method and restriction fragment length polymorphism (RFLP) (7,8). on the other hand the 18s rRNA genes have been successfully applied to identify and classify several unknown *Babesia*and*Theileria*parasites (9,10)

Materials and methods

Sample collection:

This study was carried out during September 2014 until Joan 2015 in AL-Qadisya province and it is villages. About 163 blood samples were collected from 68 goats and 95 sheep the blood was collected randomly and also from animals clinically suspected with the disease (5ml) of blood were obtained from the jugular vein and about (2ml) of blood were collected also from the ear vein the samples were put in EDTA tubes then transferred to the laboratory to make the slides and vein put in deep freezer under (20-℃) for DNA extraction.

Polymerase chain reaction method:

The PCR method was used for detection of *Babesia*and *Theileria*parsite from sheep and goats blood samples this method was carried out according to the method that described by (11).

Genomic DNA extraction and PCR amplification:

Genomic DNA was extracted from frozen blood samples by using AccuPrep®Genomic DNA extraction kit (Bioneer. Korea) and done according to instruction of company. Five micro litters (5µl) of genomic DNA was amplified in 20µl reaction mixture containing 1.5µl of each F and R primers, 12µl of DNAase water. After that, these PCR master mix component placed in standard Accu Power PCR Pre Mix kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCL PH: 9.0, KCL, MgCL2, stabilizer and tracking dye). The first round of the PCR was done according to the following program: initial denaturing step of 5min at 95℃ followed by 30 cycle of denaturing, annealing (58) for 30 sec and extension 72℃ for 40sec final extension was performed for 5min at 72℃ . PCR products were electrophoresed on 1.5% agarose gel containing ethidumpromide for visualization under UV light.

In order to differentiation between *Babesiaspp*, the semi nested PCR were carried out employing the PCR products and using P2, P3 for *Babesiaovis*detected and P2, P4 for *Babesiamotasi*detected. The technique was performed in a total volume of 20µl as in first round. The semi nested PCR thrmocycle condition include 95℃ for 5min for initial denaturation followed by 30 cycle of denaturing, annealing at 58 and extension 72 for 30sec final extension was for 10min at 72℃.

DNA sequencing and phylogenetic analysis:

Fourteen selected PCR products were purified from agarose gel by using the (EZ-10 column DNA Gel Extraction Kit) the MEGA6. Program was used to perform multiple sequence alignments. The sequence were compared with the Gene Bank data base by a nucleotide sequence homology search were compared with the others different word strains by using NCBI blast of nucleotides sets and alignment between our strains and the word strains by the using clustle W2 program online after that all word strains confirmed with the Iraqi strains. The identity score was usually analyze for building phylogenetic tree by the use of neighbor method for constriction tree (12)

Statistics analysis :

The results of the present study were analyzed by SPSS program (version 19) software 2010, using Chi-square test(X^2) and P values of $p < 0.05$ were considered to record statistical significance (13).

Results :

In order to diagnosis of *Babesis*infection five different primers were used to differentiation of *Babesisspp* ,*Babesiaovis* ,*Babesismotasi*and*Babesiscrassa*and according to PCR examination of sheep and goat DNA samples, the results showed that among (95) sheep suspected cases the positive samples were 32 (33.6%) and on the other hand ,among (68) goat suspected cases, the positive samples were 8 (11.7%) so there is significant differences between them at level ($P < 0.05$) as figure (1)

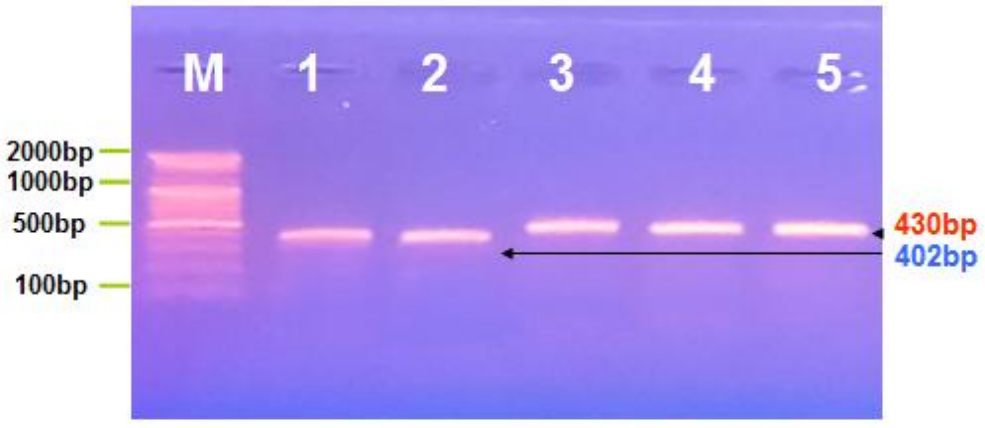

Figure (1) Agarose gel electrophoresis image that show the PCRproduct analysis of 18S rRNA gene that detection from sheep and goatsGenomic DNA samples. Where M: Marker (2000-100bp), lane(1-2)positive PCR samples (*Babesiaspp*) at 402bp product size. Lane (3-5) positive PCR samples (*Theileraspp*) at 430bp product size.

. Prevalence of Babesiosis according to the parasite different species by using semi nested PCR method in sheep and goats.

According to the parasite species differentiation by using semi nested PCR method the highest rate of infection (30%) that was observed in *Babesiaspp*, while the lowest rate infection (5%) that was observed in *Babesiamotasi*on the other hand ,the rate of *Babesiaovis*infection was(6%) as in figure (2) .

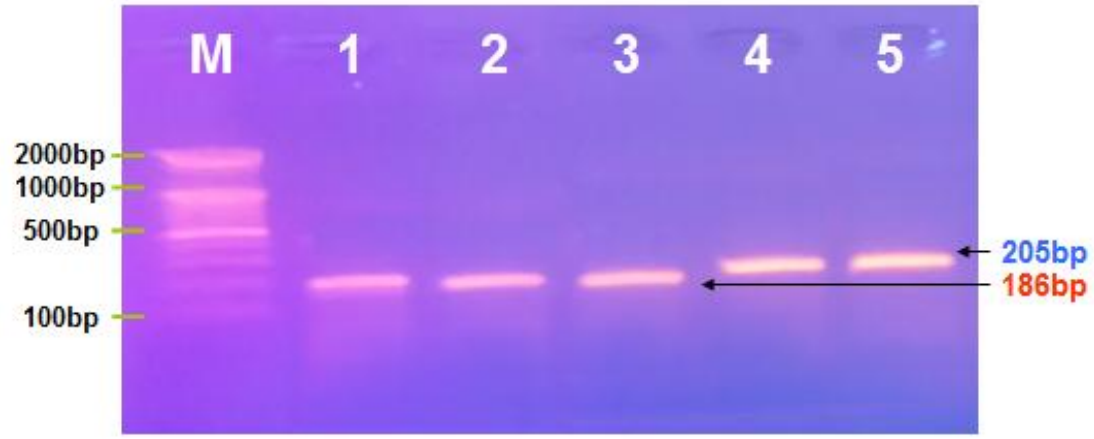

Figure (2) Agarose gel electrophoresis image that show the PCR product analysis of 18S rRNA gene in *Babesiaspp*. that detection from sheep and goats Genomic DNA samples. Where M: Marker (2000-100bp), lane(1-3) positive PCR samples (*Babesiaovis*) in sheep and goats at 186bp product size. Lane (4-5) positive PCR samples (*Babesiamotasi*) in sheep and goats at 205bp product size.

Prevalence of Babesiosis according to *Babesiacrassa*in sheep and goat by using conventional PCR method.

In order to diagnosis of *Babesiacrassa*in sheep and goat the conventional PCR method was used and among (18) suspected sheep and goat the rate of infection with *Babesiaspp* was (100%) while the rate of infection with *Babesiacrassa*was (0%) and the result was negative so there were significant differences between at level ($p < 0.05$) as in figure (3) .

Figure (3) detection of *Babesiacrassa*by PCR method through amplification of (18s) rRNA gene the lanes (1,2,3,4,5,6,7,8,9,10) negative samples (584 bp) in sheep and goat.

. Prevalence of Babesiosis in sheep and goats according to animals age.

The results showed significant differences ($P < 0.05$) in the prevalence rate among different age groups .the highest infection rate in animals of the age group (2-4) years recorded 40% (20 positive samples out of 50 total screened while the lowest infection rate (20%) recorded in animals of the age group (1-6) months alsothe results showed significant differences ($P < 0.05$) in the prevalence rate among different age groups in goatsThe highest infection rate in animals of the age group (6-12) months and (1-2) years recorded 20% while the lowest rate of infection was (3.3%) was observed in age (2-4) year so there was no infection recorded in animals of the age group (1-6) months as in table 1 and 2

Table (1) PCR positive cases in sheep according to the age

Age	Examined No.	Positive No.	Percentage (%)
1-6 month	10	2	20 B
6-12 month	13	5	38.4 A
1-2 year	22	5	22.7 B
2-4 year	50	20	40 A
Total	95	32	33.6

*** The similar letters refers to the non–significant differences while the different letters refers to the significant differences at (P < 0.05)>**

Table (2) PCR positive cases in goat according to the age

Age	Examined No.	Positive No.	Percentage (%)
1-6 month	3	0	0 C
6-12 month	20	4	20 A
1-2 year	15	3	20 A
2-4 year	30	1	3.3 B
Total	68	8	11.7

*** The similar letters refers to the non–significant differences while the different letters refers to the significant differences at (P < 0.05).**

Prevalence of Babesiosis in sheep and goats according to the animals sex.

The resultsshowed that there was non – significant differences between the six of the sheep at level (P < 0.05) so the highest rate of infection (40%) that was observed in female while the lowest rate of infection was (26.6%) that was observed in male on the other hand, the highest rate of infection in goats (12.5%) that was observed in female while the lowest rate of infection (10.7%) that was observed in male so there was non – significantly different between the six at level (P < 0.05)as in table (3)and (4) .

Table (3) PCR positive cases in sheep according to the sex

Sex	Examined No.	Positive No.	Percentage (%)
Male	45	12	26.6 A
Female	50	20	40 A
Total	95	32	33.6

Table (4) PCR positive cases in goat according to the sex

Sex	Examined No.	Positive No.	Percentage (%)
Male	28	3	10.7 A
Female	40	5	12.5 A
Total	68	8	11.7

Sequencing results: (Nucleotides sequencing sets)

Fourteen purified PCR products samples were analyzed by the use of Dye –terminator sequencing method in order to obtain the nucleotide sets of the (18s rRNA) gene (389-402 bp) that isolated from several different regions in AL-Diwaniya province . So results of the nucleotides sequence sets of this study confirmed and checked by using the NCBI – Basic Local Alignment Search Tool (BLAST analysis) by using nucleotide database within nucleotide query program online .sequences confirmation and examination were checked by using references of 18s rRNA gene of *Babesia*parasite that include both *Babesiaovis*and*Babesiamotasi* gene sequences data information that recorded in Gene Bank and the out groups to find the degrees of identity and similarity score of the 18s rRNA gene of *Babesia*species generally and *Babesiaovis* ,*Babesiamotasi*subspecies that effected the animals include sheep and

goat and compared with our isolates strains .the results of our local *Babesiaspp* (no1 ,no2 ,no3 ,no4 ,no6 ,no7 ,no8 ,no11 ,no12 ,no13 ,no14) were showed closed related to NCBI – Blast *Babesiaovis*isolates and other NCBI – Blast *Babesiaspp*were showed more different to our local *Babesiaspp*.the percentage of identity score ranged from (83 – 100%) on the other hand ,the results of our local *Babesiaspp* (no5 ,no9 ,no10) were showed closed related to NCBI – Blast *Babesiamotasii*solates and other NCBI –Blast *Babesiaspp*were showed more different to our local *Babesiaspp* .The percentage of identity score of our *Babesiamotasi*ranged from (81 -100%). After that the alignment of multiple sequence obtained by using (clustal W2) program on line as (14) .

Results of phylogenetic tree construction:

The results of our phylogeny tree and the sequences analysis of the 18s rRNA gene of *Babesiaovis*showed that *Babesiaovis* No.1(sheep-Iraq) that isolated from Daghara region in AL-Qadisiya province ,*Babesiaovis* No.10 (sheep-Iraq) ,*Babesiaovis* No.6 (sheep-Iraq) ,*Babesiaovis*No.7 that isolated from Sadeer region in AL-Qadisiya province and *Babesiaovis*No.5(sheep-Iraq) that isolated from city center were close related to *Babesiaovis* (sheep-Turkey.4) (KF 459964.1) while *Babesiaovis*No.8 (sheep-Iraq) ,*Babesiaovis*No.11 (goat-Iraq) , *Babesiaovis*No.9 (sheep-Iraq) that isolated from Sadeer region and *Babesiaovis*No.2 ,*Babesiaovis*No.3,*Babesia ovis*No.4 that isolated from Daghara region were showed close related to *Babesiaovis*(sheep-Iraq Kurdistan.1) (KC 778787.1) and also to *Babesiaovis*(goat-Tunisia) (KF 723612.1) as in figure (5).

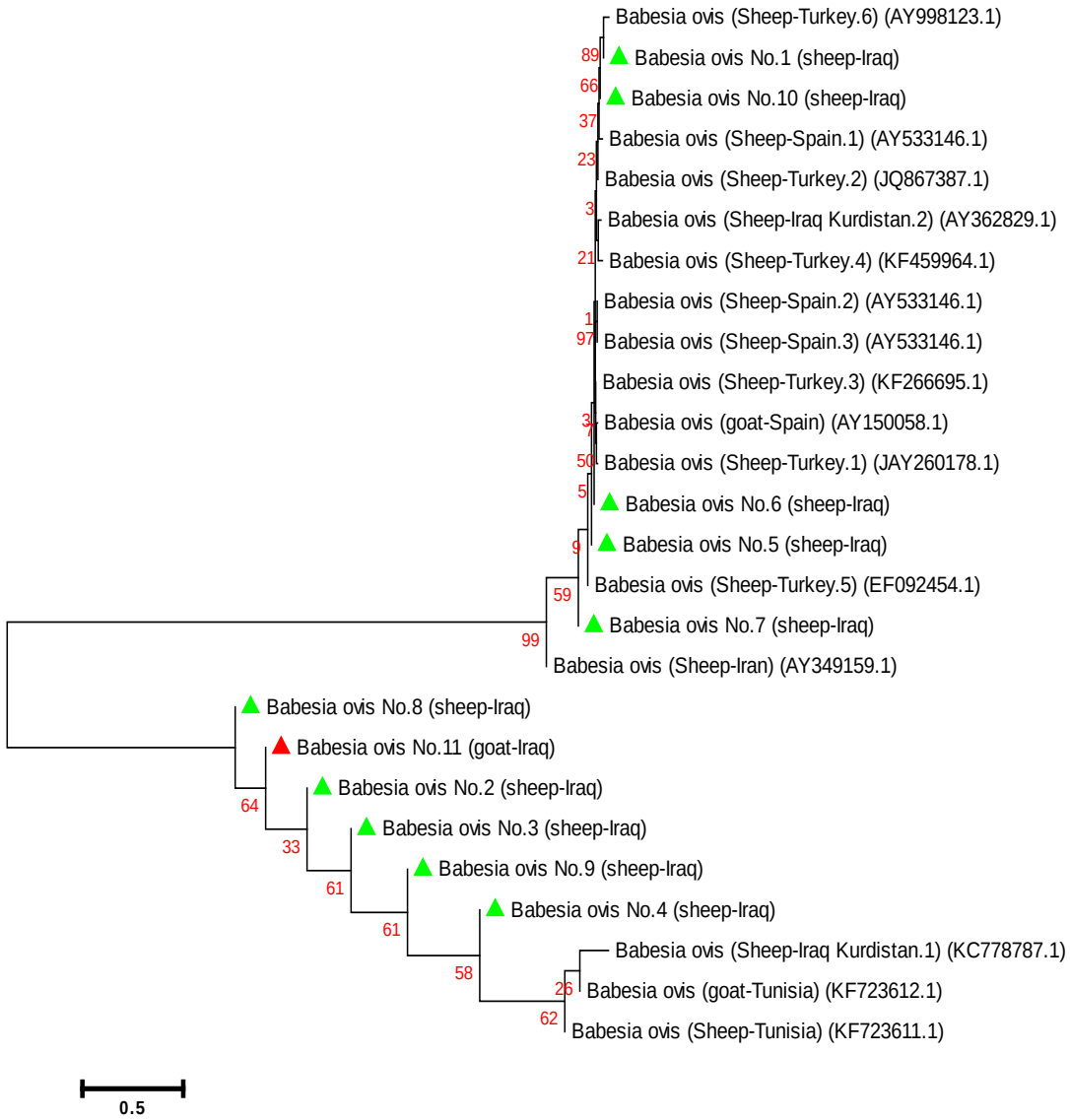

Figure (5): Phylogenetic tree analysis based on the 18S ribosomal RNA gene partial sequence that used for local *Babesiaovis* sheep and goat relationship

On the other hand ,the results of phylogeny tree of our (18s) rRNA gene of *Babesiamotasi*appeared that *Babesiamotasi*No.1(goat-Iraq) that isolated from Sadeer region ,*Babesiamotasi* No.2 (sheep-Iraq) that isolated from Daghara region were close related to *Babesiamotasi*(sheep-Netherlands) (AY 260179.1) while *Babesiamotasi*No.3 (sheep-Iraq) that isolated from Sadeer region was close related to *Babesiamotasi*(sheep-Spain) (AY 533147.1) as in figure (6).

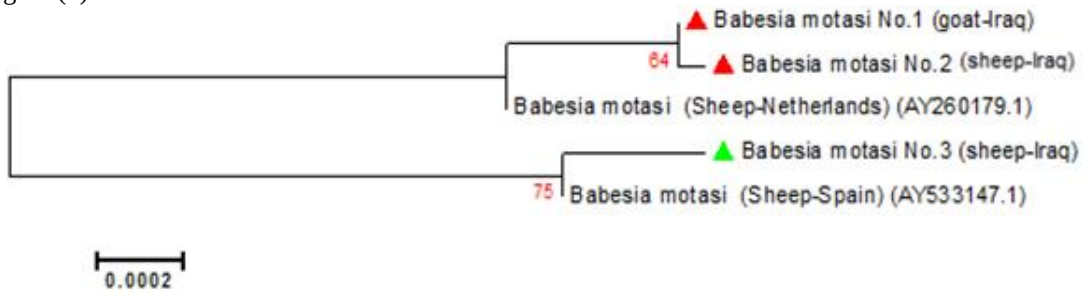

Figure (6): Phylogenetic tree analysis based on the 18S ribosomal RNA gene partial sequence that used for local *Babesiamotasi* sheep and goat relationship

Discussion :

The results showed that during the first round of semi nested PCR to differentiation between *Babesia*and*Theileria*among (95) sheep suspected cases, the positive samples were 32 (33.6%) and in goats among (68) goats suspected cases the positive samples were 8 (11.7%) While during the second round of semi nested PCR the results showed that when we examined the (40) positive samples that 18 (45%) samples out of (40) samples was detected as *Babesiaspp*, 6 (15%) samples out of (40) samples was detected as *Babesiaovis*(5 sheep samples and 1 goat samples) and 2 (5%) samples out of (40) samples was detected as *Babesiamotasi*(1 sheep sample and 1 goat sample) so the current study showed that the rate of *Babesiaovis*infection was more than *Babesiamotasi*infection according to semi nested PCR results with significant differences at level ($P < 0.05$) so our data did not confirm with the Iraqi studies such as (15on the other hand ,the results of (16) was lower than our results because they found that about 3 (1.5%) was positive samples for *Babesiaovis*out from 195 sheep that examined by PCR while the result was negative when the samples were examined by RLB. also our results was nearly to the finding(17) when he and other sciences pointed to that according to the semi nested PCR examinationexamination*Babesiaovis*infection rate was about to (14%) while they don't found *Babesiamotasi*during their examination so they considered *Babesiaovis*as a main causative agent of the disease in sheep in east Azerbaigan in Iran. In our study we also use the conventional PCR to detected *Babesiacrassa*by the use of specific forward and reverse primers and about 18 (45%) samples out of 40 samples that include 12 sheep and 6 goats were examined and the result was negative. so our data was fit with the finding of (16) when they recorded a low rate of *Babesiaovis*infection but did not found *Babesiacrassa* in Kurdistan region in north of Iraq. Our results about *Babesiacrassa*also fit with (18) when they did not found *Babesiacrassa*during their examination for 20 peripheral blood samples of sheep that was suspicion to Babesiosis but they found large *Babesia*infective sheep which morphologically similar to *Babesiamotasi*but genetically it identical to *Babesiaovis*in study carried out in Iran

In the current study and according to the age of sheep and goats our results showed that there was significant differences at level ($P < 0.05\%$) in rate of infection among different age groups In related to sex of animals, our results showed that there was non- significant differences at level ($P < 0.05$) between the sex of sheep and goats so this agreement with the finding of (19) when he referred to that there was no differences observed in *Babesiaovis*infected between male and female .

On the other hand, our data of the phylogeny tree and the sequences analysis of the 18s rRNA gene of *Babesiaovis*were appeared that *Babesiaovis*No1 (sheep-Iraq) under the accession number (KP998109.1) , *Babesiaovis*No10 (sheep-Iraq) (KR264960.1) ,*Babesiaovis*No6 (sheep-Iraq) (KR264954.1), *Babesiaovis*No7 (sheep-Iraq) (KR264955.1) ,*Babesiaovis*No5(sheep-Iraq) (KR264953.1) were showed highest homology with the world strain under the accession number (KF459964.1) that isolated from sheep Turkey.4

While other our isolate that include *Babesiaovis*No8 (sheep-Iraq) (KR264958.1), *Babesiaovis*No11 (goat-Iraq) (KR264961.1), *Babesiaovis*No9 (sheep-Iraq) (KR264959.1), *Babesiaovis*No2 (sheep-Iraq) (KP998110.1), *Babesiaovis*No3 (sheep-Iraq) (KP998111.1) and *Babesiaovis*No4 (sheep-Iraq) (KP998112.1) showed high similarity

and identity with *Babesiaovis*(sheep-Iraq) that isolated from Kurdistan region and under the accession number (KC778787.1) and with *Babesiaovis*(goat) they isolated from Tunisia (KF723612.1) . On the other hand, the results of our phylogenetic tree of 18s rRNA gene of *Babesiamotasi*showed that *Babesiamotasi*No1 (goat-Iraq) (KP998113.1), *Babesiamotasi*NO2 (sheep-Iraq)(KR264956.1) showed close relationship with the strain of *Babesiamotasi*that isolated from Netherlands sheep (AY260179.1) while *Babesiamotasi*No3 (sheep-Iraq) (KR264957.1) appeared high homology and identity with *Babesiamotasi*strain that isolate from (Spain-sheep) (AY533147.1)

References

:

1-Shayan, P.andRahbari S.(2007). Differentiation of sheep *Theileriaspp.*and*Babesia spp*. by Polymerase Chain Reaction, J.Vet.Res. 62(2):15-20

2-Telford 3rd, S.R.; Gorenflot, A.;Brasseur, P.; Spielman, A.(1993). Babesial infection in human and wildlife. In: Kreier, J.P., Baker, J. R. (Eds.), Parasitic Protozoa, vol. 5, 7. Academic Press, San Diego, pp.1-47.

3-Kakoma, I. and Mehlhorn, M.(1994).*Babesia* of domestic animals. In: Kreier, J. P. (Ed.), Parasitic Protozoa, vol. 7. Academic Press, San Diego, pp. 141-216.

4-Bock, R.; Jackson, L.; Devos, A. and Jorgensen, W. (2004) . Babesiosis of cattle. Parasitology. 129: 247-269

5-Ahmed, J.; Yin, H.; Schnittger, L. and Jongejan, F. (2002). Ticks and tick-borne diseases in Asia with special emphasis on
China. Parasitol Res 88:S 51-S 55.

6-Tavasoli, M. and Rahbari, S. A(1998). Seroepidemiological survey on *Babesiaovis* in different geographical region of Iran.Vet J Tehran University. 53 (3,4): 55-59.

7-Blears, M. J.; Pokorny, N.J.; Carreno, R. A.; Chen, S.; De Grandis , S. A. and Lee , H., *etal*., (2000). DNA finger printing of *Cryptosporidium parvum*isolatesusing amplified fragment length polymorphism (AFLP). J. Parasitol., 86 (4): 838-841.

8-Guy, R. A.; Xiao, C. and Horgen, P. A. (2004). Real time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. J ClinMicrobiol.; 42 (7): 33 17-20.

9-Persing, D.H.;Herwaldt, C.; Glaser, R.S.; Lane, J.W.; Thomford, D.;Mathiesen,P.J.;Krause,D.E.;Philip and Conrad, P.A.(1995) Infection with *Babesia* like organism in northern of California .New Journal of Medicine .;332:298-303 .

10-Gubbels, M. J.; Homg, Y.; Van, D.; Weide, M.; Qi, B.; Nijman, I. J.; Guangyuan, L. and Gongejan, F. (2000). Molecular characterisation of the *Theileriabuffeli* / orientalis
group .International Journal for parasitology. 30(8), 943-952.

11-Shayan, P. and Rahbari, S. (2005). Simultaneous differentiation between *Theileriaspp* and *Babesiaspp*on stained blood smear using PCR, Parasitol Res. 97: 281-286.
.

12-Tamura, K. ; Dudley, J. ; Nei, M. and Kumar, S.(2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

13-Leech, N. L., Barrett, K. C. and Morgan, G. A. (2011). IBM SPSS for Intermediate statistics. 4th ed. Taylor and Francis Group . LLC. USA.

14-Goujon, M.; McWilliam, H.; Li, W.; Valentin, F.; Squizzato, S.; Paern, J.and Lopez, R. *(*2010).Clustal W2 A new bioinformatics analysis tools framework at EMBL-EBI .Nucleic acids research, W695-9*doi:10.1093/nar/*gkq313

15-Sulaiman, E. G.; Arslan,S.H.; Al-Obaidi,Q.T. and Daham, E.(2009). Clinical, haematological and biochemical studies of babesiosis in native goats in Mosul. Iraq. J. Vet. Sci. 24, 31-35.

16-Renneker, S.; Abdo, J.; Bakheit, M. A.; Kullmann, B.; Beyer, D.; Ahmed, J. and Seitzer, U. (2012).coinfection of sheep with *Anaplasma* ,*Theileria*and*Babesia*species in the Kurdistan region, Iraq. Blackwell verlagGmbh. Transboundary and Emerging Diseases .60(Suppl.2) 113-118.

17-Shahbazi, A.; Fallah, E.; Mamagani, A. J.; Khanmohammadi, M.; Nematollahi, A.; Bazmani, A. (2013). Identification of *Babesia*species in sheep isolated from villages of east Azerbaijan by semi -nested PCR. Journal of pure and applied microbiology, vol. 7(1), p. 201-205.

18-Shayan, P. and Nabian, S. (2008). The Use of Immunostaining for Determination of *Babesia*and*Theileira* and Gene Expression of Proliferation Associated with Nuclear Protein in *Theileria*Infected Cells .Iran Vet J 3 (3): 21-29

19-Rjeibi, M. R.; Gharbi, M.; Mhadhbi, M.; Mabrouk, W.; Ayari, B.; Nasfi, I.; Jedidi, M.; Sassi, L.; Rekik, M. and Darghouth, M. A. (2014). Prevalence of piroplasms in small ruminants in north-west Tunisia and the first genetic characterisation of *Babesiaovis*in Africa .published by EDP sciences, parasite, 21-23.

.