مجلة الدراسات التربوية والعلمية - كلية التربية - الجامعة العراقية
العدد الثالث والعشرون - المجلد التاسع - علوم الحياة - آب 2024 م
doi.org/10.52866/esj.2023.09.23.29

First Hematological and Molecular Detection of *Anaplasma phagocytophilum* in Donkeys in Iraq.

Mohanad Mohammed jameel[1] , Saleem A. Hasso[2]
[1] Department of Veterinary Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
dr.mhnd84@gmail.com
[2]Department of Veterinary Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
saleemaminhasso@yahoo.com

Abstract:

Anaplasma phagocytophilum is a multi-host pathogen that causes granulocytic anaplasmosis. The aim of the study was to determine the percentage of donkeys chronically infected with *A. phagocytophilum*, molecular detection , the DNA sequencing and phylogenetic tree analysis of this bacterium. A total of 100 donkeys were examined ,100 blood samples were collected and examined by blood smear and by nested PCR using primers specific for *16 SrRNA* and *msp4* genes. The phylogenetic tree was built in MEGA 6.0 using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree). The Results showed that the blood smear tested under microscope out of 100 samples 7(7%) were positive for the (morula).In Nested PCR out of 100 sample 14(14%) were positive for *16 SrRNA* and 10(10%) for *msp4* . The DNA sequencing and the phylogenetic tree showed that the isolate *A. phagocytophilum* was closely related to the NCBI-BLAST *A.phagocytophilum* Hungary isolate at total genetic changes of (0.1%) .

Keywords: *Anaplasma phagocytophilum*, donkeys, hematology,Nested PCR, Iraq.

الكشف الدموي والجزيئي الاول عن انابلازما فاجوسايتوفيلم في الحمير في العراق

مهند محمد جميل / فرع الطب الباطني والوقائي البيطري ، كلية الطب البيطري، جامعة بغداد، بغداد،
dr.mhnd84@gmail.com
سليم امين حسو / فرع الطب الباطني والوقائي البيطري ، كلية الطب البيطري، جامعة بغداد،
saleemaminhasso@yahoo.com

مستخلص

الأنابلازما البلعمية هو مُـمْرِض متعدد المضيف يسبب داء الأنابلازما المحبب. هدفت الدراسة إلى تحديد نسبة الحمير المصابة بشكل مزمن ببكتيريا الأنابلازما البلعمية والكشف الجزيئي وتسلسل الحمض النووي وتحليل شجرة النشوء والتطور لهذه البكتيريا. تم فحص 100 حمار وجمع 100 عينة دم وفحصها عن طريق مسحة الدم وتفاعل البوليميراز المتسلسل المتداخل باستخدام بادئات خاصة بـ جين *SrRNA16* و *msp4*. تم بناء شجرة النشوء والتطور في MEGA 6.0 باستخدام طريقة المجموعة الزوجية غير الموزونة مع المتوسط الحسابي (شجرة UPGMA). أظهرت النتائج أن مسحة الدم التي تم اختبارها تحت المجهر من بين 100 عينة، 7 (7٪) كانت إيجابية لـ (التوتية). في Nested PCR من 100 عينة، كانت 14 (14٪) إيجابية لـ *16SrRNA* و 10 (10٪). بالنسبة لـ *msp4* . أظهر تسلسل الحمض النووي وشجرة النشوء والتطور أن العزلة الأنابلازما البلعمية كانت مرتبطة بشكل وثيق بعزلة المركز الوطني لمعلومات التكنولوجيا الحيوية باستخدام طريقة تسلسل الحمض النووي الأنابلازما البلعمية المجر عند تغيرات جينية إجمالية قدرها (0.1٪).

كلمات مفتاحية : *الأنابلازما البلعمية* ، البغال ، دموي ، تفاعل البلمرة المتسلسل المتداخل ، العراق .

Introduction

The newly authorized term *Anaplasma phagocytophilum* replaces three granulocytic bacterial species: *Ehrlichia phagocytophila, Ehrlichia equi*, and the agent of *human granulocytic Ehrlichiosis*, following a reorganization of the families Ricketttsiaceae and Anaplasmataceae under the order Ricketsiales (1). The bacterium *Anaplasma phagocytophilum* affects humans, horses, domestic ruminants, dogs, cats, and ticks and causes granulocytic anaplasmosis. equine granulocytic anaplasmosis (EGA) was originally identified as a horse illness in California (1, 2) and has subsequently spread to other regions of the United States , Europe and South America (3,4). The intensity of the clinical symptoms varies,fever, anorexia, apathy, leg oedema, and petechial hemorrhages are common symptoms (5). In laboratory diagnosis (leukopenia, anemia, thrombocytopenia, and identification of intracytoplasmic inclusion bodies in leukocytes) and clinical findings (fever, anorexia, lethargy, limb oedema, and petechial hemorrhages) are used to diagnose *A. phagocytophilum* in animals in the acute phase (6). The earliest laboratory diagnosis for acute infection is blood smear; the presence of the distinctive cytoplasmic inclusion bodies in neutrophils and, less frequently, eosinophils in blood smears is diagnostic (7). *A.phagocytophilum* DNA can be detected by PCR in unclotted blood or Buffy coat smears and DNA may also be detected in host blood using a sensitive and specific PCR. (8). Previous studies conducted on *A. phagocytophilum* by another researcher's for identification it on another animals and those researchers are:(9). This study was directed to investigate the prevalence of *Anaplasma phagocytophilum* in Iraq and samples were taken from cattle, (10) The study was designed to record the percentage of infection and presence of *A. phagocytophilum* infection within sheep and ticks in Iraq, (11) The study was designed to record First detection of equine anaplasmosis and hemoplasmosis of horses in mosul city, Iraq, and another study was by (12) The study was designed to record the percentage of infection and presence of canine anaplasmosis (*A. phagocytophilum and A.platys*) in dogs in Iraq.

The aim of the present study was to determine the percentages of clinically and chronically infected carrier's don-

keys for *A. phagocytophilum*, at the molecular level in donkeys by using nested PCR fallowed by DNA sequencing *and* phylogenetic tree analysis.

Materials and Methods:

Sampling: one hundred donkeys (Equus asinus) from different places Erbil and Duhok in Iraq were use in this study in which 100 blood samples were taken aseptically. Blood was collected from the animals via jugular vein puncture with test tubes containing (EDTA) anticoagulant. These samples were used for screening the presence of *A. phagocytophilum* by making thin film slides of blood smear was diagnosed based on blood abnormalities and the discovery of *A. phagocytophilum* organisms (morula) in blood. Positive and negative samples were subjected to DNA extraction.

DNA extraction:

Blood genomic DNA was isolated from the frozen blood samples according to the manufacturer's instructions using the gSYAN DNA extraction kit (Geneaid, USA). The extracted genomic DNA was examined with a Nanodrop spectrophotometer (THERMO. USA), to assess DNA purity by measuring absorbance at 260 /280 nm.

PCR Reaction

Molecular characterization by using conventional PCR for detection of *A. phagocytophilum*. The Green PCR Master Kit (Promega, USA) was used in the PCR reaction. Tap DNA polymerase, dNTPs (dATP, dCTP, dGTP, dTTP) Tris-HCl pH 9.0, KCl, $MgCl_2$,Stabilizer and Tracking dye. PCR thermocycler (T100 Thermal cycler PCR Bio-Rad/ USA) settings were accomplished using fallowing program: PCR program for 16srRNA: 35 time (denaturation at 95ºC 30sec , Annealing at 55ºC 30sec, Extension at 72ºC1min) . Nested PCR program for *16srRNA*: 35 time (Denaturation at 95ºC30sec , Annealing at 55ºC30sec, Extension at 72ºC1min). PCR program for *msp4* : 35 time (Denaturation at 95ºC30sec , Annealing at 54ºC30 sec, 72ºC1min). Nested PCR program for *msp4* : 35 time (Denaturation at 95ºC30sec , Annealing at 55ºC 30sec, Extension at 72ºC1min). Agarose gel electrophoresis was used to examine the Nested PCR results, it was made by dissolving 1X TBE in a water bath, then stained with 3µl of ethidium bromide stain, 10µl of PCR product was put to each comb well, along with 3µl of (100bp Ladder) in the first well. The

electric current was then applied for 1 hour at 100 volts and 80 ampere, UV Transilluminator was used to visualize nested PCR products s . The positive amplified genes sequences with specific ladder marker (100-2000bp) to specify the molecular weight of amplified genes sequences which appear as bands. DHL delivered the nested PCR product of *Msp4* gene positive items to Macrogen Company in Korea in an ice bag for DNA sequencing using the AB DNA sequencing technology DNA sequencing was performed using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA 6.0) and Multiple sequence alignment analysis of the incomplete Msp4 genes based on ClustalW alignment analysis. The evolutionary distances were determined using the phylogenetic tree UPGMA technique and the Maximum Composite Likelihood method.

Table 1: Primers used in 1st and 2ed run of nested PCR

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	Reference
16SrRNA	F	CACATGCAAGTCGAACGGATTATTC	(932bp)	(13)
	R	TTCCGTTAAGAAGGATCTAATCTCC		
16SrRNA nested PCR primers				
	F	AACGGA TTATTCTTTA TAGC TTGCT	(546bp)	
	R	GGCAGTATTAAAAGCAGCTCCAGG		
MSP4	F	ATGAATTACAGAGAATTGCT TGTAGG	(849bp)	(14, 15)
	R	TTAATTGAAAGCAAATCTTGCTCCTATG		
MSP4 Nested PCR primers				
	F	CTATTGGYGGNGCYAGAGT	(381 bp)	
	R	GT TCATCGAAAATTCCGTGGTA.		

Statistical Analysis: The current study's data were statistically analyzed using the Statistical Package For Social Sciences version 28. The chi-square test was done to determine the relationship between the variable percentages. To compare the mains of two groups at a 95% confidence interval (p0.05), descriptive statistics and an independent t-test were utilized to examine clinical

and hematological variables. Test results were considered statistically significant if their P value was less than 0.05 at the level of significance. 2016 (16).

Results

Clinical signs: All of the donkeys in the study clinically assessed for temperature, respiration, and heart rate, were normal, while The infection was persistent in those animals, varied clinical symptoms were detected, including emaciation and a pale mucosal membrane.

Infection rates

Infection rates based on blood smear staining

The presence of morula in the cytoplasm of granulocytes during blood smear testing is strongly predictive of a diagnosis(figure 1). For the first time, A. phagocytophilum was found in the blood of a donkey suffering from poor performance, weight loss, and anemia. The percentage of positive cases of blood smear in microscopic examination were 7(50%) out of 14(100%) positive samples (table 1) in PCR test out of 100 donkeys. the result of blood smear in infected donkeys was significant at $p \leq 0.001$.

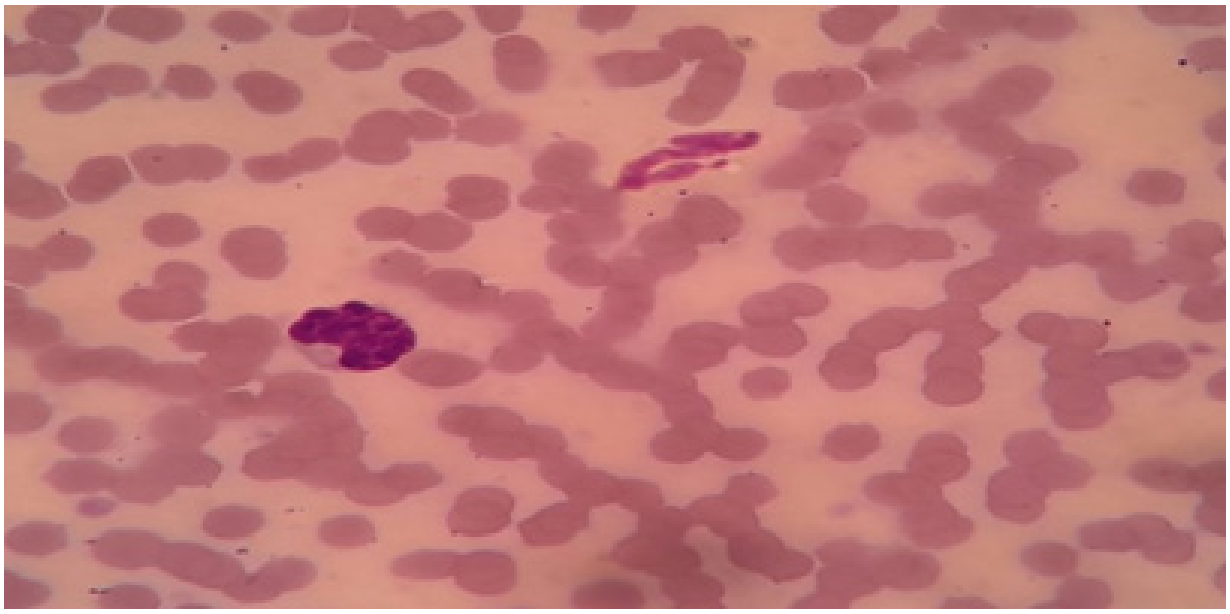

Figure 1: The morula in the blood of donkeys.

Table 2: Distribution of positive blood smears in donkeys.

		Blood Smear		Total
		Positive	Negative	
infected	Count	7	7	14
	% of infection	50 %	50 %	100 %
Non-infected	Count	0	86	86
	% of infection	0 %	100 %	100 %
Total	Count	7	93	100
	% of infection	7%	93 %	100 %
Pearson Chi-Square		0.000*		

Infection rate in Molecular detection

The Nested PCR product analysis of the 16S RNA gene in A. phagocytophilum from donkey blood samples, where M represents the DNA marker and Lanes (1-14) only Positive samples at (546bp) (figure 2), the percentage of infected animal was 14 (14%) and 86 was Non-infected out of 100 donkeys (table 3). the result of nested PCR test of 16SrRNA gene in *A. phagocytophilum* in infected donkeys was significant at $p \leq 0.001$.

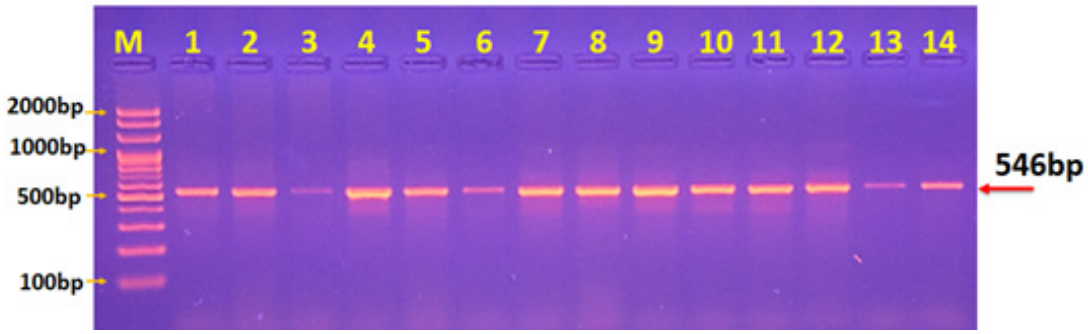

Figure (2) The Nested PCR product analysis of the 16S RNA gene in *A. phagocytophilum* from donkey blood samples.

Table 3:Distribution of positive *16SrRNA* gene in donkeys.

		16SrRNA gene		Total
		Positive	Negative	
infected	Count	14	0	14
	% within infection	100 %	0 %	100 %
Non-infected	Count	0	86	86
	% within infection	0 %	100 %	100 %
Total	Count	14	86	100
	% within Infection	14 %	86 %	100 %
Pearson Chi-Square		0.000*		

Nested PCR product analysis of the *msp4* gene in *A. phagocytophilum* blood samples, M stands for marker (2000-100bp). Lanes (1-14) only had a few positive samples at the (381bp) (figure 3)Nested PCR product. Table3 shows the positive samples in nested PCR test of msp4 gene of *A. phagocytophilum* isolates from donkeys blood samples and the percentage of infected animals was 10 (10%) out of 100 donkeys. the result of nested PCR test of *msp4* gene in *A. phagocytophilum* in infected donkeys was significant at p≤ 0.001.

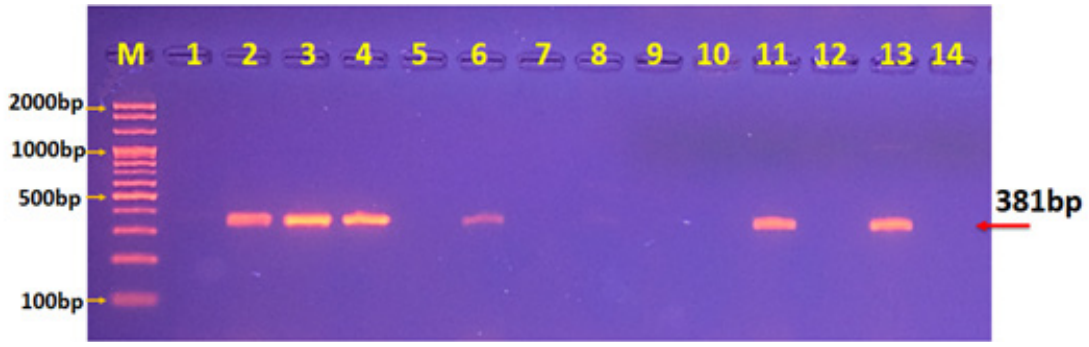

Figure (3): Nested PCR product analysis of the *msp4* gene in *A. phagocytophilum* blood samples.

Table 4 : Distribution of *msp4* gene positive cases in donkeys.

		msp4 gene		Total
		Positive	Negative	
infected	Count	10	4	14
	% within infection	71.4 %	28.6 %	100 %
Non-infected	Count	0	86	86
	% within infection	0 %	100 %	100 %
Total	Count	10	90	100
	% within infection	10 %	90 %	100 %
Pearson Chi-Square		0.000*		

DNA Sequence results:

The examination of the phylogenetic tree revealed that *A. phagocytophilum* isolate from the donkey were closely related to NCBI-BLAST *A.phagocytophilum* the Hungary isolates at total genetic changes of 0.1%. The genetic homology sequence identity between *A. phagocytophilum* isolates from donkeys and NCBI-Genbank deposited related *A. phagocytophilum* Hungary isolates ranged from 99.21-99.23%, and the phylogenetic tree similarity was 97%, as shown in Table (4). Finally, two isolates of A. phagocytophilum from donkey were successfully registered within the NCBI GenBank and assigned accession numbers (OP244687.1-and OP244688.1).

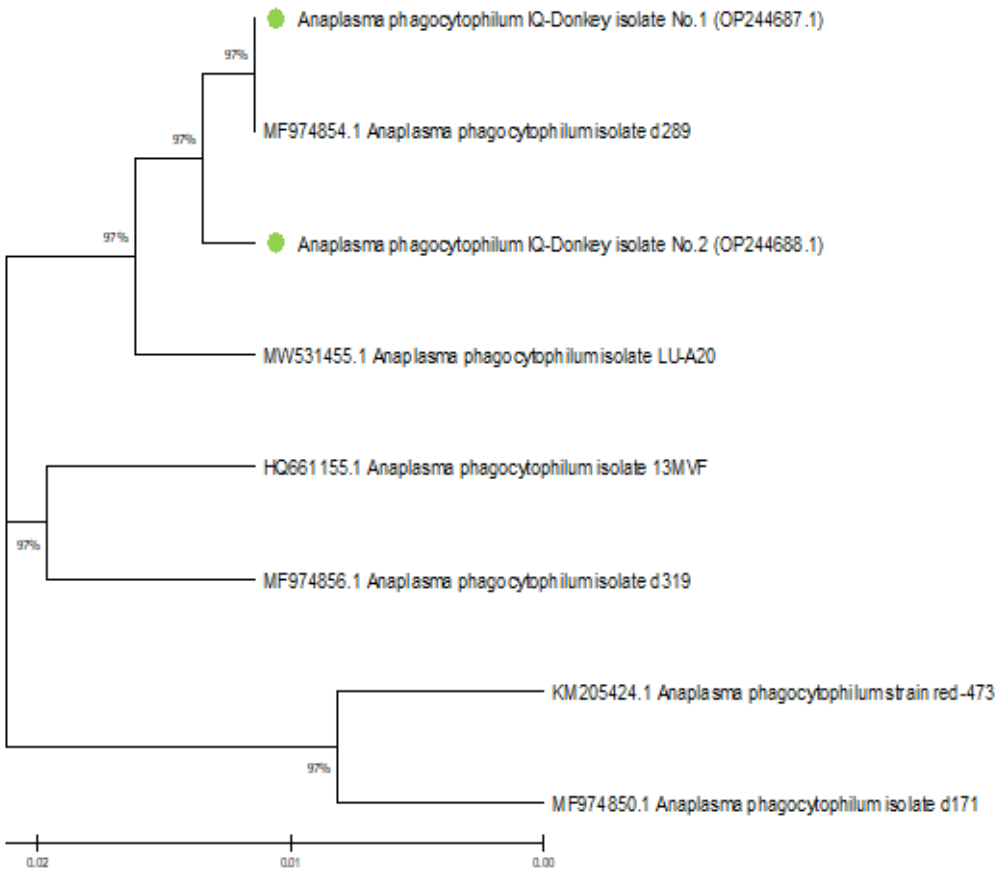

Figure 4: Phylogenetic tree analysis based on the partial sequence of the *msp4* gene in local A. phagocytophilum donkey isolates

The phylogenetic tree was built in MEGA 6.0 using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) for genetic relationship investigation at total genomic alterations (0.1%), *A. phagocytophilum* donkey isolate was found to be closely related to the NCBI-BLAST *A. phagocytophilum* Hungary isolate. Table 4 displays the percentage of NCBI-BLAST Homology Sequence identity between local *A. phagocytophilum* donkey isolates and NCBI-BLAST closely related isolates of. *A. phagocytophilum* in other countries. The result show that the tree similarity for the first isolate was 96% and identity 99.21% with the Hungary isolate, the tree similarity for the second isolate was 96% and identity 99.23% with Hungary isolate.

Table 5: The percentage of NCBI-BLAST Homology Sequence identity between local *A. phagocytophilum* donkey isolates and NCBI-BLAST closely related isolates .

A. phagocytophilum isolate	Accession number	Homology sequence identity (%)			
		Identical *A. phagocytophilum*	Accession number	Similarity %	Identity (%)
Anaplasma phagocytophilum IQ-Donkey isolate No.1	OP244687.1	*Anaplasma phagocytophilum* d289 Hungary isolate	MF974854.1	96%	99.21%
Anaplasma phagocytophilum IQ-Donkey isolate No.2	OP244688.1	*Anaplasma phagocytophilum* d289 Hungary isolate	MF974854.1	96%	99.23%

Discussion

A. phagocytophilum causes granulocytic ehrlichiosis in various species of domesticated mammals, including equids. According to this study, *A. phagocytophilum* infects donkeys in northern Iraq. The donkeys in this investigation did not exhibit clinical signs, which was consistent with previous findings. (17-18). This could be due to a number of factors such as infection phase (acute or carrier), immunological status, age, infective dose, environment, and management. This makes them chronically infected carriers and keeps their clinical symptoms

under control (13). In this investigation, *A. phagocytophilum* organisms were detected microscopically in Iraqi donkeys. The anaplasma's intracellular replication results in the formation of these morulae. The findings demonstrated that *A. phagocytophilum* obviously replicates within neutrophils. The presence of morulae in neutrophil cytoplasmic vacuoles in blood smears is considered diagnostic for ehrlichiosis. (19-20) and the results were agree with another study (10). on donkey in (Sicily- Italy) . *A. phagocytophilum* molecular characterization and utilization of the 16SrRNA gene and the result in this study were agree with previous study conducted by (21 in Italy and22 in Spain) for detection of *A. phagocytophilum* in donkey. The diagnosis of A. phagocytophilum by using *msp4* gene and result agree with study of (21) in Italy through the number of animals in the survey and through the number of positive infections. The *msp4* gene is expressed on the outer membrane of *A. phagocytophilum*, this gene product is thought to be involved in the host pathogen interaction, and may evolve more rapidly than other nuclear gene proteins, and thus may show host specific characteristics due to selective pressures exerted by the host immune systems, resulting in a high sequence heterogeneity among *A. phagocytophilum* strains in this particular gene (9;10) Separate clustering in ruminants is another example of evolution connected to host sensitivity and geographical distribution of this organism (10). The ability to distinguish *A. phagocytophilum* samples based on their mammalian host of origin suggested that msp4 sequences could be employed for coevolutionary investigations (10). The msp4 sequences of the MRK isolate, which was originally isolated from a horse in California, and the Italian strains from donkeys were identical (10).

Conclusion:

It can be concluded from the result that the chronic form of the disease was prevalent, the acute form was not recorded. *A. phagocytophilum* was recorded for the first time in the donkeys in Iraq. The use of specific primer for nested PCR test was extremely specific and sensitive than microscopic examination.

Acknowledgment

The author is grateful to the University of Baghdad /College of Veterinary Medicine for all the facilities to achieve this study.

Conflict of interest

There is no conflict of interest.

References:

1. Dumle JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray S C, etal.,. Reorganization of genera in the families' *Rickettsiaceae* and Anaplasmataceae in the order *Rickettsiales*; unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichiaequi* and "HGE agent"as subjective synonyms of *Ehrlichia phagocytophila. Int.J.Syst.Evol. Microbiol*. 2001; 51, 2145–2165. doi: 10.1099/00207713-51-6-2145.
2. Gribble D.H. Equine ehrlichiosis. J Am Vet Med Assoc, 1969, 155:462–469.
3. Woldehiwet Z. The natural history of *Anaplasma phagocytophilum. Vet. Parasitol*. 2010 ; 167, 108–122. doi: 10.1016 /j.vetpar.2009.09.013.
4. Madigan, JE. Equine ehrlichiosis. Vet Clin North Am Equine Pract. 1993; 9:423–428.
5. Thomas RJ, Dumler JS, Carlyon JA. Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and Ehrlichia ewingii ehrlichiosis. Expert Rev Anti Infect Ther, 2009; 7:709–722.
6. Bakken JS. and Dumler JS. Human granulocytic anaplasmosis. Infect. Dis. *Clin. NorthAm*. 2008; 22, 433–448.doi: 10.1016 /j.idc..03.011.
7. Elias E. Diagnosis of ehrlichiosis from the presence of inclusion bodies or morulae of *E. canis*. J. Small Anim. Pract.1991;.33,540-543.
8. Rassouli M and Aghazamani G. Retrospective Study of Tick-Borne Pathogens and Observation of Ehrlichia ewingii/Anaplasma phagocytophilum and Hemotropic Mycoplasma spp. in Dogs' Blood Films. 2015.
9. Ajel,B.K and mahmood ,A.K.(2021). Polymerase Chain Reaction Study For Anaplasma Phagocytophilum In Iraqi Cows . Sys Rev Pharm 2020;11(11):1961-1966.

10. Karrar Jasim Hamzah Al-Janabi, Saleem Ameen Hasso(2019). Detection of Anaplasma phagocytophilum infection in sheep in some provinces of Iraq. Al-Qadisiyah Journal of Veterinary Medicine Sciences, Volume 18, Issue 1, Pages 73-80.
11. Albadrani, B.A, Al-Iraqi, O.M. (2019). First detection of equine anaplasmosis and hemoplasmosis of horses in mosul city, iraq. Adv. Anim. Vet. Sci. 7(2):106-111.
12. Obaidi, S. S. A., Khalaf Al-Ani, J. M., & Al-Shammari, N. B. (2020). Molecular Detection of Some Anaplasma Species in Blood of Dogs in Baghdad Province, Iraq. The Iraqi Journal of Veterinary Medicine, 44(1), 39–45.
13. Massung RF, Slater K, Owens JH, Nicholson WL, Mather TN, Solberg VB, Olson JG. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol. 1998;36(4):1090–5.
14. de la Fuente J, Massung RB, Wong SJ, Chu FK, Lutz H, Meli M, von Loewenich FD, Grzeszczuk A, Torina A, Caracappa S, Mangold A.J, Naranjo V, Stuen S, Kocan KM. Sequence analysis of the msp4 gene of *Anaplasma phagocytophilum* strains. J. Clin. Microbiol. 2005 ; 43, 1309–1317.
15. Bown KJ, Lambin X, Ogden NH, Petrovec M, Shaw SE, Woldehiwet Z, Birtles RJ. High-resolution genetic fingerprinting of European strains of *Anaplasma phagocytophilum* by use of multilocus variable-number tandem-repeat analysis. J Clin Microbiol. 2007;45(6):1771–6.
16. George D, and Mallery P.IBM SPSS Statistics 23 Step by Step: A Simple Guide and Reference (14th ed.). 2016;New York: Routledge. https:// doi.org/ 10.4324 / 9781315545899.
17. Kumar S, Kumar R., Sugimoto C. A perspective on *Theileria equi* infections in donkeys. Jpn. J. Vet. Res. 2009;56 (4) 171–180.
18. Veronesi F, Morganti G, Ravagnan S, Laus F, Spaterna A, Diaferia M, Moretti A, Fioretti D P, Capelli G. Molecular and serological detection of tick-borne pathogens in donkeys (Equus asinus) in Italy. Veterinary Microbiology. 2014;173 , 348–354.
19. Taylor S M and Kenny J. The effects of tick-borne fever (*Ehrlichia*

phagocytophila) on the growth rate of fattening cattle. Br. Vet. J.1980; 136,364–370.

20. Center for Food Security and Public Health, College of Veterinary Medicine .Iowa State University. Institute for International Cooperation In Animal Biologics. Ehrlichiosis Available at:http://www-cfsph.Iastate.edu\ehrlichiosis Accessed 1May 2005.
21. Torina A, Alongi A, Naranjo V, Scimeca S, Nicosia S, Di Marco V, *etal*. Characterization of *Anaplasma* infections in Sicily, Italy. *Ann. N.Y.Acad.Sci*.2008b; 1149, 90–93. doi: 10.1196/annals.1428.065.
22. Naranjo V, Ruiz-Fons F, Hofle U, FernandezDeMera IG, Villanua D, Almazan C. Molecular epidemiology of human and bovine anaplasmosis in southern Europe. *Ann.N.Y. Acad.Sci*. 2006 ;1078, 95–99.doi: 10. 1196/ annals. 1374. 013.