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First microscopic and molecular detection of *A. phagocytophilum* in mules in Iraq

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Abstract

Anaplasma phagocytophilum, a multi-host pathogen, causes granulocytic anaplasmosis. A total of 50 mules were examined by collecting 50 blood samples by forming blood smears. All sample were examined under a light microscope and then conformed using conventional nested PCR utilizing specific primers for *16SrRNA* and the *msp4* gene and partial sequence *msp4* gene for *A. phagocytophilum*. The genetic connection study for the gene in *A. phagocytophilum* mules isolates and NCBI-Genbank related *A. phagocytophilum* country isolates was performed using DNA sequencing. The phylogenetic tree was built in MEGA 6.0 using the Unweighted Pair Group Method with Arithmetic Mean. The Results showed that the infection was persistent in those animals, with varied clinical symptoms including emaciation and a pale mucosal membrane in infected mules. Body temperature, respiration and heart rate were average. The clinical signs of the positive samples varied although some infected animals had no clinical indications. The blood smear examination of 50 samples showed that 4 (8%) were positive and showed the morula. The nested PCR for *A. phagocytophilum* genes (*16 SrRNA* and *msp4*) showed that 8(16%) were positive for *16SrRNA* and 7(14%) for the *msp4* gene. The sequence analysis of *A. phagocytophilum* isolates via NCBI-BLAST showed a closed relatedness to *A. phagocytophilum* isolate of Hungary at total genetic changes (0.1%).

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Introduction

The bacterium *A. phagocytophilum* affects humans, horses, domestic ruminants, dogs, cats, and ticks and causes granulocytic anaplasmosis. Following a rearrangement of the families *Rickettsiaceae* and *Anaplasmataceae* under the order *Ricketsiales*, it replaced three species of granulocytic bacteria, *Ehrlichia phagocytophila* (ruminants), *Ehrlichia equi* (horses), and the agent of human granulocytic ehrlichiosis (1). Equine granulocytic anaplasmosis (EGA) was initially identified as a horse illness in California (2) and has subsequently spread to other regions of the United States and Europe South America (3,4). The disease's occurrence is influenced by the genetic variety of *A. phagocytophilum*, the host involved, and the vectors prevalent in a given location (5,6). Common symptoms include high fever, lethargy, partial anorexia, staggering or ataxia, distal limb edema, and hematological abnormalities such as thrombocytopenia, neutropenia, lymphopenia, and mild anemia (7). Clinical and laboratory symptoms (leukopenia, anemia, thrombocytopenia, and identification of intracytoplasmic inclusion bodies in leukocytes) are used to diagnose *A. phagocytophilum* in animals in the acute phase (3). Early-stage acute infection can be identified by the distinctive cytoplasmic inclusion bodies in neutrophils (8). A sensitive and specific PCR for *A. phagocytophilum* DNA detection in host blood has been created. PCR can detect *A. phagocytophilum* DNA in blood smears (9).

The current study aimed to determine the percentage of chronically infected carrier's mules (*Equus asinus* × *Equus*

caballus) by *A. phagocytophilum*. The molecular detection of *A. phagocytophilum* in mules using conventional PCR. DNA sequencing of *A. phagocytophilum and* Phylogenetic tree analysis.

Materials and methods

Ethical approve

Approval for this study was obtained from the committee of College of Veterinary Medicine/ University of Baghdad, Iraq. Number 39/PG on 7/1/2021.

Sampling

50 blood samples from 50 mules were taken aseptically. Blood was drawn from the animals through jugular vein punctures in tubes containing the anticoagulant (EDTA). These samples were used for detecting the presence of *A. phagocytophilum* by making thin film slides of blood smears. Positive and negative samples were submitted to DNA extraction molecular characterization by using conventional Nested PCR for detection of *A. phagocytophilum*. The amplified DNA was sequenced to confirm the detection of local *A. phagocytophilum*.

DNA extraction

DNA was isolated from blood samples according to the manufacturer's instructions using the gSYAN DNA kit extraction kit (Frozen Blood) Geneaid (Taiwan) using a Nanodrop spectrophotometer (THERMO. USA) for checking DNA quantity by measuring absorbance at (260/280 nm).

PCR Reaction

The Green PCR Master Kit was used to create the PCR master mix prepared according to the manufacturer's recommendations. PCR thermocycler settings were accomplished using a typical PCR thermocycler system: 5 minutes of denaturation at 95°C, denaturation at 95C° for 30 sec, annealing at 55 C°30sec, extension at 72C°1min-5 minutes at 72 C°, final hold is at 4 C°. Nested PCR findings were examined using agarose gel electrophoresis. Agarose gel electrophoresis was used with red safe dye with a specific ladder marker (100-2000bp) to specify the molecular weight of amplified genes sequences which appear as bands. Multiple sequence alignment analysis of the missing *Msp4* genes based on ClustalW alignment analysis was used in conjunction with DNA sequencing using Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0). The Maximum Composite Likelihood approach and the UPGMA algorithm on phylogenetic trees were used to calculate the evolutionary distances (Table 1).

Statistical analysis

The data from the current study 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. Descriptive statistics and an independent t-test were utilized to examine clinical and hematological variables to compare the mains of two groups at a 95% confidence interval ($P<0.05$). Test results were considered statistically significant if their P value was less than 0.05 at the significance level (13).

Table 1: Primers and PCR scripts for identifying *A. phagocytophilum*

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	Program	Reference
16SrRNA	F	CACATGCAAGTCGAACGGATTATTC	932	I	10
	R	TTCCGTTAAGAAGGATCTAATCTCC			
16SrRNA nested PCR primers	F	AACGGATTATTCTTTATAGCTTGCT	546	II	
	R	GGCAGTATTAAAAGCAGCTCCAGG			
MSP4	F	ATGAATTACAGAGAATTGCT TGTAGG	849	1	11,12
	R	TTAATTGAAAGCAAATCTTGCTCCTATG			
MSP4 Nested PCR primers	F	CTATTGGYGGNGCYAGAGT	381	2	
	R	GTTCATCGAAAATTCCGTGGTA			

PCR program: I: 35 time (95ºC30sec, 55ºC30sec, 72ºC1min). II: 35 time (95ºC30sec, 55ºC30sec, 72ºC1min).1: 35 time (95ºC30sec, 54ºC30sec, 72ºC1min). 2: 35 time (95ºC30sec, 55ºC30sec, 72ºC1min).

Results

The infection rate in blood smear

All of the mules in the study were clinically assessed. The infection was persistent in those animals, and varied clinical symptoms were detected in those infected with *A. phagocytophilum*, including emaciation and a pale mucosal membrane in the infected mules. Temperature, respiration, and heart rate were average. varied clinical signs in infected animals, and some infected animals had no clinical indications. This study, *A. phagocytophilum* was identified by looking for the organisms (morula) in buffy coat smears and blood samples. Blood smear testing for morula in granulocyte cytoplasm significantly predicts a diagnosis. For the first time, *A. phagocytophilum* was found in the blood of anemic, underperforming, and losing weight mules. For the

first time in Iraq, blood samples from mules were examined for *A. phagocytophilum* (Figure 1 and Table 2).

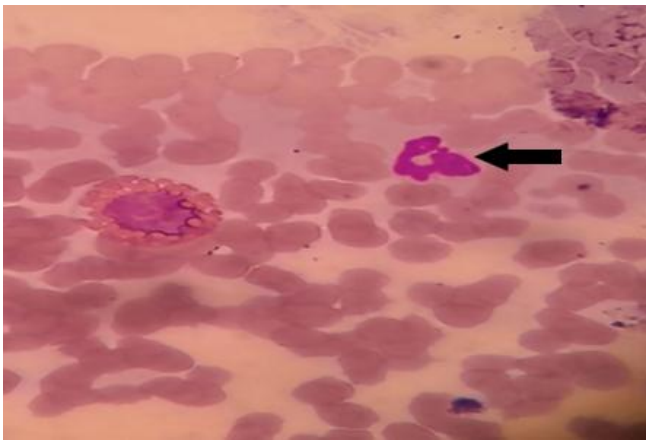

Figure 1: Shows the morula in the blood of mules.

Table 2: Distribution of positive blood smear in mules

Blood smear		+ve	- ve	Total
Infected	Count	4	4	8
	% within	50.0	50.0	100.0
Non-Infected	Count	0	42	42
	% within	0.0	100.0	100.0
Total	Count	4	46	50
	% within	8.0	92.0	100.0
Pearson Chi-Square p			0.000*	

The percentage of positive cases of blood smear in microscopic examination was 4(50%) out of 8 (100%) positive samples by PCR test out of 50 mules. The blood smear result in infected mules was significant at P≤ 0.001.

Molecular detection

Nested PCR was used as a detection technique for *A. phagocytophilum* studied gens (16 SrRNA and mgsp4) to visualize the positive amplified gene sequences (Figures 2 and 3, Tables 3 and 4).

DNA Sequence results

The genetic relationship study of the *Msp4* gene in *A. phagocytophilum* isolates and NCBI-Genbank relatedness *A. phagocytophilum* country isolates was performed using DNA sequencing. The examination of the phylogenetic tree (genetic connection) revealed that *A. phagocytophilum* mule isolates showed a close relation to NCBI-BLAST *A. phagocytophilum* Hungary isolates at total genetic changes 0.1%. The homology sequence identity between *A. phagocytophilum* mule isolates and NCBI-Genbank related *A. phagocytophilum* Hungary isolate showed a genetic homology sequence identity range 99.21%. furthermore, the phylogenetic tree similarity was 96%. Finally, *A. phagocytophilum* mule isolates were submitted to NCBI Genbank and identified by accession numbers OP244689.1-OP244690.1 (Figure 4 and Table 5).

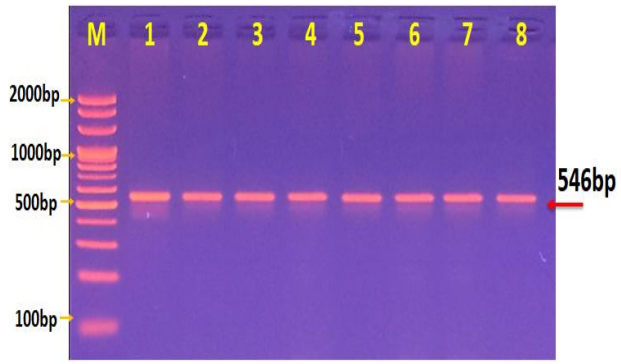

Figure 2: The results of the *16Sr RNA* gene in *A. phagocytophilum* blood samples are shown. Lanes (1-8) showed only Positive samples at (546bp) Nested PCR product where M: marker.

Table 3: Distribution of positive *16S RNA* gene in mules

16SrRNA gene		+ve	- ve	Total
Infected	Count	8	0	8
	% within	100.0	0.0	100.0
Non-Infected	Count	0	42	42
	% within	0.0	100.0	100.0
Total	Count	8	42	50
	% within	16.0	84.0	100.0
Pearson Chi-Square p			0.000*	

This table shows the positive samples in the nested PCR test of the *16S RNA* gene in *A. phagocytophilum* from mule's blood samples. The percentage of infected animals was 8 (16%) and 42 were non-infected out of 50 mules. The result of the nested PCR test of the *16SrRNA* gene in *A. phagocytophilum* in infected mules was significant at P≤ 0.001.

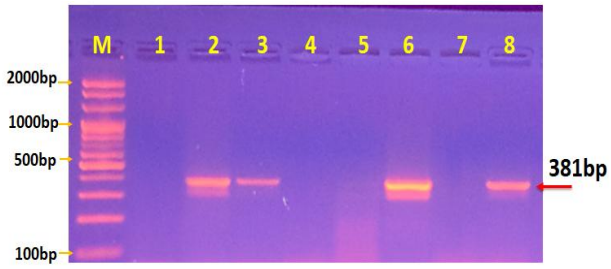

Figure 3: The msp4 specific gene in *A. phagocytophilum* from mule by Nested PCR. Lanes 1-8 and M represent positive samples and DNA markers (100 - 2000bp) respectively. The product size was 381bp.

Table 4: Distribution of *msp4* gene positive cases in mules

msp4 gene		+ve	- ve	Total
Infected	Count	7	1	8
	% within	87.5	12.5	100.0
Non-Infected	Count	0	42	42
	% within	0.0	100.0	100.0
Total	Count	7	43	50
	% within	14.0	86.0	100.0
Pearson Chi-Square p			0.000*	

This table shows the positive samples in nested PCR test of the *msp4* gene in *A. phagocytophilum* from mule's blood samples and the percentage of infected animals was 7 (14%) and 43(86%) were non-infected out of 50 mules. The result of nested PCR test of the *msp4*gene in *A. phagocytophilum* in infected mules was significant at P≤ 0.001.

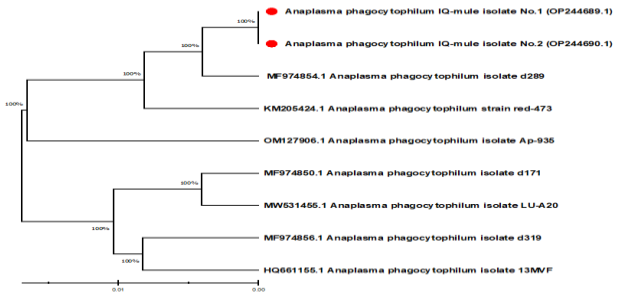

Figure 4: *Msp4* gene partial sequence analysis in local *A. phagocytophilum* mule isolates used for genetic connection analyses. At total genetic alterations (0.1%), the *A. phagocytophilum* mule isolates were found to be closely related to the NCBI-BLAST *A. phagocytophilum* isolate Hungary isolate.

Table 5: The proportion of NCBI-BLAST Homology Sequence identity between local *A. phagocytophilum* mule isolates and NCBI-BLAST closed genetic related *A. phagocytophilum* country isolates

A. phagocytophilum isolates	Accession number	Homology sequence identity (%)			
		Identical *A. phagocytophilum*	Number	Similarity	Identity
A. phagocytophilum IQ-mule isolate No.1	OP244689.1	*A. phagocytophilum* d171 Hungary isolate	MF974850.1	96%	99.21%
A. phagocytophilum IQ-mule isolate No.2	OP244690.1	*A. phagocytophilum* d171 Hungary isolate	MF974850.1	96%	99.21%

Phylogenetic tree study based on the partial sequence of the (Msp4) gene in a local *A. phagocytophilum* mule isolate used for genetic connection analysis. The phylogenetic tree was built in MEGA 6.0 using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree). At total genomic alterations 0.1%, the *A. phagocytophilum* mule isolate was closely related to the NCBI-BLAST *A. phagocytophilum* isolate Hungary isolate.

Discussion

A. phagocytophilum causes granulocytic ehrlichiosis in various domesticated mammals, including equids. *A. phagocytophilum* is endemic or potentially endemic in 42 countries of the world. This has been detected throughout Europe, America (North and South), Asia (Pakistan, India, Korea, and Japan) and Africa (14-21). Human seroprevalence in disease endemic area of Wisconsin and New York (USA) is 15-36%, whereas seroprevalence in Europe range from 1 and 20% depending upon immunity, tick exposure, and age of the patients (22). Majority of the human cases of infection in USA occur in June-July.

This study showed that *A. phagocytophilum* infects mules in the north of Iraq. The mules in this study did not show clinical symptoms, and This outcome was consistent with what others had reported (23,24). This could be due to several 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 (25).

In this investigation, *A. phagocytophilum* organisms were discovered microscopically in Iraqi mules, the microscopic examination of blood smears is an easy, rapid, and cheap field test that used in the diagnosis of *A. phagocytophilum* (26,27). The anaplasma's intracellular replication results in forming these morulae. The morula observed in the cytoplasmic vacuoles of white blood cells specially neutrophils in blood smears, is considered diagnostic for ehrlichiosis (11), and the results agreed with another study Saleem *et al.* (28) on mules in Pakistan, Torina *et al.* (29) on donkeys in Sicily- Italy, Naranjo *et al.* (30) in Spain, Yousefi *et al.* (31) on dogs in Iran, Atif (32) and Taylor *et al.* (33). This method has low sensitivity, in cases of chronic, subclinical, or persistent infection. Therefore, the results of the microscopic examination method should be confirmed using more sensitive and accurate techniques such molecular techniques (34).

The molecular characterization of *A. phagocytophilum* and the use of 16SrRNA gen and the results of this study agree with previous studies conducted by Saleem *et al.* (28), Torina *et al.* (29), Naranjo *et al.* (30), Yousefi *et al.* (31) for the detection of *A. phagocytophilum* in donkeys and other

animals. The diagnosis of *A. phagocytophilum* using the msp4 gene and results agreed with the study Saleem *et al.* (28), Torina *et al*. (29), Naranjo et al (30). MSP4 is expressed on the outer membrane of *A. phagocytophilum*. *MSP4* is thought to be involved in host-pathogen interaction and may evolve more rapidly than other nuclear gene proteins, resulting in host-specific characteristics due to selective pressures exerted by host immune systems, resulting in high sequence heterogeneity among *A. phagocytophilum* strains in this particular gene (10,11). Separate clustering in ruminants is another example of evolution connected to host sensitivity and geographical distribution of this creature (11).

The ability to distinguish *A. phagocytophilum* samples based on their mammalian host of origin suggested that *msp4* sequences might be employed for coevolutionary research (11). The msp4 sequences of the MRK isolate, which was initially isolated from a horse in California, and the Italian strains from donkeys were identical (11). phylogenetic analysis of the msp4 sequences differentiated between strains of *A. phagocytophilum* from humans, dogs, and horses from those obtained from ruminants (35). This study and other studies have shown that *A. phagocytophilum* and many blood parasites infects mules and many animals like small and large ruminants and the infection spreads in the north and middle of Iraq (35).

Conclusion

It can be concluded from the result that the chronic form of the disease was prevalent, but the acute form was not recorded. *A. phagocytophilum* was recorded for the first time in the mules in Iraq. The use of specific primers for nested PCR tests was particular and sensitive to microscopic examination.

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Conflict of interest

There is no conflict of interest.

Editorial board note

Saleem A. Hasso is member of the editorial board of the Iraqi Journal of Veterinary Sciences, he did not participate in any stage of the decision-making process for this article.

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الكشف المجهري والجزيئي الأول عن الانابلازما البلعمية في البغال في العراق

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الخلاصة

تسبب الأنابلازما البلعمية، وهي مسببات الأمراض متعددة المضيف، داء الأنابلازما المحبب. تم فحص ما مجموعه ٥٠ بغلاً مع جمع ٥٠ عينة دم عن طريق تشكيل مسحات دموية وتم فحص جميع العينات تحت المجهر الضوئي ومن ثم تأكيدها باستخدام تفاعل البلمرة المتسلسل المتداخل التقليدي باستخدام بادئات محددة لـجين الحمض النووي الرايبوزي الرايسومي ١٦ وجين البروتين السطحي الرئيسي ٤ للتسلسل الجزئي لـ الأنابلازما البلعمية. تم إجراء دراسة الارتباط الوراثي لجين البروتين السطحي الرئيسي ٤ في عزلات الأنابلازما البلعمية للبغال وعزلات بلد الأنابلازما البلعمية المرتبطة بـ المركز الوطني لمعلومات التكنولوجيا الحيوية باستخدام طريقة تسلسل الحمض النووي. تم بناء شجرة النشوء والتطور باستخدام طريقة المجموعة الزوجية غير الموزونة مع المتوسط الحسابي. وأظهرت النتائج أن العدوى كانت مستمرة في تلك الحيوانات، مع أعراض سريرية متنوعة بما في ذلك الهزال والغشاء المخاطي الشاحب في البغل المصاب. وكانت درجة حرارة الجسم والتنفس ومعدل ضربات القلب طبيعية. وتباينت العلامات السريرية للعينات الإيجابية، رغم أن بعض الحيوانات المصابة لم تظهر عليها أي مؤشرات سريرية. أظهر فحص مسحات الدم لـ ٥٠ عينة أن ٤ منها (٨%) كانت إيجابية وأظهرت التويتة. أظهر تفاعل البوليميراز المتسلسل المتداخل لجين الأنابلازما البلعمية لجين الحمض النووي الرايبوزي الرايسومي ١٦ وجين البروتين السطحي الرئيسي ٤، أن ٨ (١٦%) كانت إيجابية لجين الحمض النووي الرايبوزي الرايسومي ١٦ و٧ (١٤%) لجين البروتين السطحي الرئيسي ٤، وأظهر تحليل تسلسل عزلة الأنابلازما البلعمية عبر المركز الوطني لمعلومات التكنولوجيا الحيوية- أداة تقصي التراتب الموضعي الأساسي وجود علاقة وثيقة لعزلة الأنابلازما البلعمية من المجر في التغيرات الوراثية الكلية (٠,١%).