Research Article

Proceeding of 8th International Scientific Conference, College of Veterinary Medicine University of Basrah, Dec. 7-8, 2022,Iraq.

BASRAH JOURNAL OF VETERINARY RESEARCH, 2022, 21(S1):44-57
https://bjvr.uobasrah.edu.iq/

Clinical, Biochemical and Molecular study of *Mycoplasma haemocanis* in Dogs in Southern Provinces of Iraq

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Accepted: Nov. 2022

Abstract

The present study aimed to detection and identified the infection 0f *Mycoplasma haemocanis* in dogs through clinical manifestation, blood film, conventional PCR technique and sequences analysis. The total number of dogs examined clinically were one hundred, and 25 clinically healthy control in southern Iraq provinces. Examined dogs showed various clinical signs. Blood samples were taking for hematological and biochemical analysis. 72 (72%) of blood smear were positive for *Mycoplasma haemocanis*, whereas 33(45.8%) positive based on conventional PCR technique. hematological examination in infected animals showed a significant decrease in mean of TRBC, Hb and PCV and significant increase ($p<0.05$) in the TLC. biochemical examination indicates a significant increase in AST, ALP and ALT in infected animals. The local *Mycoplasma haemocanis* were showed genetic variant related to NCBI-BLAST *Mycoplasma haemocanis* Turkey, Thailand, Brazil, and India isolates. In conclusion *Mycoplasma haemocanis* have been diagnosed in dogs in Basrah city with various clinical manifestation from in apparent to sever anemia, emphasized the infection using conventional PCR technique, and Phylogenetic analysis confirm the identification of *Mycoplasma haemocanis* as a new submission of local Iraq, with 99% identical to India, Brazil, Thailand and Turkey DNA sequence.

Key words: *Mycoplasma haemocanis*, signs, PCR.

Introduction

Canine haemoplasma infection is caused by *Mycoplasma haemocanis* and *Hemotropic mycoplasmas*, which characterized as cell wall-deficient bacteria that are found in a variety of animals (1) It was reclassified from *Haemobartonella canis*, and infection has been identified in both immunocompetent and immunocompromised patients (2,3). Varies from single to pairs and occasionally coccoid chains forming rings (4,5). Blood-feeding arthropods, especially the *Rhipicephalus sanguineus* (Dog ticks), are thought to spread *M. haemocanis*. However, it can be transmitted by oral ingestion or direct blood inoculation and a potential materno-fetal transplacental transmission has also been discovered (6).

The brown dog tick is widespread in Mediterranean sub-Mediterranean climate areas and the high prevalence of canine haemoplasma infections in these countries supports the theory that it is a potential tick vector for infection transmission (7,8).

The adhesion of these hemoparasites to erythrocytes results in direct damage to the membrane and consequently in reducing its life span (9), clinical signs of the disease are not clear or unspecified (10,11). The clinical signs of acute disease include infertility, fever, lethargy, anorexia, weight loss, and hemolytic anemia which may lead to death in extreme cases (11,12). Acute infection characterize by present clinical with parasitemia ,which were verified in the stained blood smear, which could vary with different degrees of illness(13). Most dogs infected with hemoplasmas are having chronic asymptomatic agents or in latent infection. In this form of infection microorganisms are found only periodically and in low numbers in the blood (14).

There is a little information about canine hemomycoplasma in dogs, so present study conducted to detection and identification of infection with *Mycoplasma haemocanis* in dogs in Basrah and other southern Iraqi provinces using different diagnostic methods.

Materials And Methods

Ethical approval: All of the experimental procedures involving animals were conducted gently and humanely during blood sampling and clinical examination.

Animals of the study

Present study conducted to include 100 of dog suspected infected with *Mycoplasma haemocanis* and 25 of clinically healthy dogs used as control group, aged between one and ten years in different breed from different southern provinces of Iraq (Basrah, Dhi Qar, Maysan and Muthanna) during the period from August to November 2020.

Clinical examination

All doges which suspected infected with *Mycoplasma haemocanis* exhibition to physical examination include body temperature, heart and respiratory rate, abnormality signs were recorded.

Samples collection

Blood samples were collected from dogs' cephalic vein,10 ml of blood sample divided to two parts,5ml put in EDTA tube for blood smear preparation and Giemsa staining

according to the standard procedure (15), used for identification of RBC infected with *Mycoplasma haemocanis.* Also was used to determine **c**omplete blood count (CBC) using an automated blood analyzer, and remind blood with anticoagulant used for conventional PCR technique. Second part 5ml of blood put in gel tubes for serum separated use in estimation the levels liver enzymes such as Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Tests were performed depending on the instruction of Cham Switzerland kit and using BA-88A biochemical analyzer (16).

Molecular Diagnosis

Blood DNA Extraction, whole blood dog samples submit extract blood DNA using the G-spinTM Total DNA Extraction Kit (INTRON, Korea).

PCR primers for direct detection *Mycoplasma haemocanis* were designed in this study using NCBI Genbank sequence database (MN294708.1) and the primer were synthesized by (Scientific researcher Co.Ltd., Iraq (table 1).

Table 1. Oligonucleotide primer used for amplification of the Mycoplasma haemocanis

16s RNA gene

Primers		Sequence (5'-3')	Product size
16S ribosomal RNA gene PCR primer	F	CTACGGGAAGCAGCAGTAGG	620bp
	R	CCTTGGTAAGGTTTTTCGTGTAT	

DNA Sequencing

The study of genetic variation between local *Mycoplasma haemocanis* isolates and Basic Local Alignment Search Tool (BLAST) program, of Country isolates was carried out using the DNA sequencing process. Three positive PCR 16SrRNA gene products were sent to Macrogen Company in Korea via DHL in a by fallow cold transport protocol DNA sequencing using an AB DNA sequencing device the evolutionary distances were calculated using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method

Statistical Analysis

Data were analyzed using SPSS (version 14.0), one way ANOVA were used to determine the significance between variances, value $P<0.05$ considered statistically significant.

RESULTS

Result of present study reveal that 72 (72%) of blood samples of dogs which susceptible infected with *Mycoplasma*

haemocanis based on microscopically examined of blood smears which exhibited various clinical signs, whereas 33(45.8%) positive based on conventional PCR technique and sequences analysis. clinically infected animals showed different clinical manifestations which include partial or complete loss of appetite, pale mucous membranes, and some animals showed congestion mucus membranes which detected on conjunctival also rapid and difficult respiration, in addition other animals were suffering from, lethargy weight loss and presence of ticks on the different parts of animal's body In addition, a significantly increase in body temperature, respiratory and heart rate in infected animals compared with the control group (Table 2).

Table2. Mean and standard deviation of body temperature, respiratory and heart rate of diseased dogs and control group.

Parameters	**Controls Mean ±SD (N=25)**	**Diseased dog Mean ±SD (N=33)**	**P –value**
Body Temperature C°	38.7 ± 0.14	39.55 ±0.33	**0.004**
Respiratory Rate/ Mint	25.2 ±2.30	35.30 ±1.92	**0.001**
Heart Rate/ Mint	86.6 ±8.24	122.6±6.98	**0.003**

Values are significant (P<0.05).

In this study microscopic examination of blood smears from dogs suspected infected with M. *haemocanis* showed coccoid or rod shape, on the erythrocyte cell wall appear individually or in chains.

Result of hematological examination indicate a significant decrease, (P<0.05) in total erythrocytes count (TEC) 4.9±0.7, hemoglobin concentration (Hb) 10.8±1.3 and packed cell volume (PCV) 38.06±7.22 compare with control and there is a significant increase (P<0.05) in mean corpuscular volume (MCV) 94.9±23.3and significant decrease (P<0.05) mean corpuscular hemoglobin (MCH) 20.9±2.3and mean corpuscular hemoglobin concentration (MCHC) 31.12±3.5 compare with control, since it reflected macrocytic hypochromic anemia, and the results leukocyte indicated a significant increase (P<0.05) in total leukocytes count (TLC) 17.93±3.4 compare with control group (Table 3).

Table3:-Mean and Standard Deviation of Blood Parameter of Infected Dog *Mycoplasma haemocanis* compared with controls.

Parameters	Controls Mean ±SD (N=25)	Infected Dog Mean ±SD (N=33)	P-Value
TEC ($\times 10^6$)	6.8 ±0.49	4.9±0.7	0.003
Hb (g/dl)	15.3±1.6	10.8±1.3	0.002
PCV (%)	49.62±7.04	38.06±7.22	0.002
MCV (fl)	70.4±2.8	94.9±23.3	0.004
MCH pg	23.2±1.1	20.9±2.3	0.003
MCHC g/dl	34.3±1.07	31.12±3.5	0.002
TLC 10^3/µL	8.4±1.18	17.93±3.4	0.004

Values are mean. (P<0.05)

Results of liver enzymes in infected dog showed there is significance difference (P<0.05) were encountered between infected dog with *Mycoplasma haemocanis* and control group since results indicated increase value of alkaline phosphates (ALP) 143.54±10.48, aspartate aminotransferase **(**AST) 29.31±10.15 and alanine aminotransferase,(ALT) 42.2±9.35 (Table 4). Result indicated that high prevalent rate of infection in Basrah province 11 (44%), While in Dhi Qar was 9 (36%), in Maysan was 7 (28) and in Muthana was 6(24%) (Table 5). Results of PCR amplification, PCR product of the targeted 16S ribosomal RNA gene in extracted *Mycoplasma haemocanis* genome that the target gene was detected in 620 bp compared with DNA marker (1500 bp) and negative control (Figure 1). Phylogenetic analysis, sequencing of 16SrRNA gene was performed to confirm the identification of *M. haemocanis* defected during the current study new submission of local Iraq, identify present study show successfully record of *Mycoplasma haemocanis* with Gen Bank accession number sequences alignment the DNA sequencing method was performed to study the determination of genetic variation between native *Mycoplasma haemocanis* isolates and NCBI BLAST Country isolates. Three PCR 16SrRNA gene positive products were sent to Macrogen in Korea in for DNA sequencing by the AB DNA sequencing system. It was found that the three samples that were sent are 99% identical to India, Brazil, Thailand and Turkey DNA sequence analysis was performed using Molecular Evolutionary Genetic Analysis version 6.0. (Mega 6.0) (Figure 2,3, Table 6).

Table 4 : Mean and standard deviation of liver enzyme of infected dog with *Mycoplasma haemocanis* compared with control group.

Parameters	Controls Mean ±SD (N=25)	Infected Dog Mean ±SD (N=33)	P –value
ALP *(U/L)*	68.32±17.22	143.54±10.48	0.003
AST *(U/L)*	14.80±2.87	29.31±10.15	0.001
ALT *(U/L)*	34.96±8.78	42.2±9.35	.004٭

Values are mean. (P<0.05)

Table5: Prevalence rate of infection with My*coplasma haemocanis* in Southern Iraqi provinces.

Province	Numbe of Dogs	Number of infection	Percentage	P-value
Basrah	25	11	44%	0.067
Dhi Qar	25	9	36%	
Mysan	25	7	28%	
Almuthna	25	6	24%	

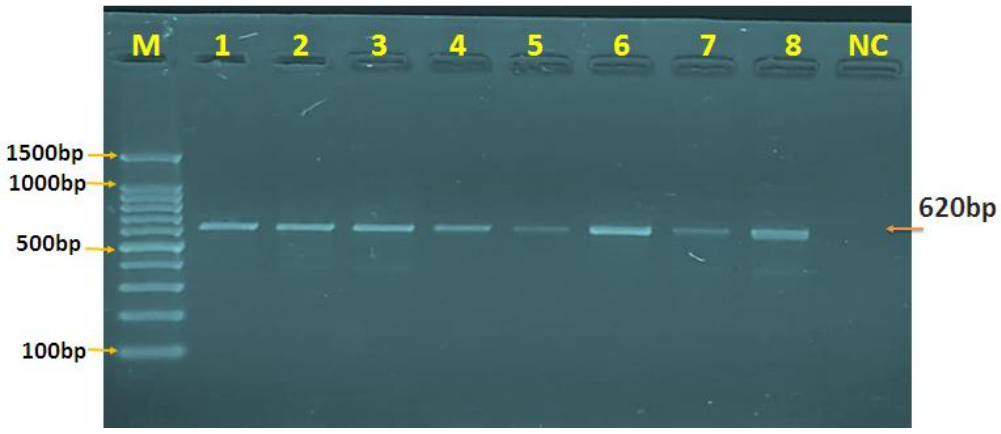

Fig. 1. Agarose gel electrophoresis image that showed the PCR product analysis of 16S ribosomal RNA gene in *Mycoplasma haemocanis* from extracted DNA of blood dog's samples. Where M: marker (1500-100bp) and the Lane (1-8) positive *Mycoplasma haemocanis* samples at (620bp) PCR product.

Mycoplasma_haemocanis_isolate1
TTAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
EU442623.1_Brazil
TAAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
Mycoplasma_haemocanis_isolate2
TTAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
MG594501.1_Turkey
TAAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
KU765208.1_Thailand
TAAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
Mycoplasma_haemocanis_isolate3
TTAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
MG050153.1_India
TAAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCA
****

Fig. 2. Multiple sequence alignment analysis of 16S ribosomal RNA gene in local *Mycoplasma haemocanis* dog isolates and NCBI-Genbank *Mycoplasma haemocanis* country related isolates. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That showed alignment analysis of nucleotide similarity as (*) and substitution mutations in 16S ribosomal RNA gene between isolates.

Table 6. The NCBI-BLAST homology sequence identity between local *Mycoplasma haemocanis* isolates and NCBI-BLAST country submitted *Mycoplasma haemocanis* Isolate

***Mycoplasma haemocanis* Isolate**	**Accession Number**	**Homology Sequence Identity (%)**			
		India	**Brazil**	**Thailand**	**Turkey**
***Mycoplasma haemocanis* No.1**	MW784616	99.30%	99.04%	99.05%	99.05%
***Mycoplasma haemocanis* No.2**	MW784617	99.30%	98.61%	98.62%	98.62%
***Mycoplasma haemocanis* No.3**	MW784618	99.07%	98.84%	98.85%	98.85%

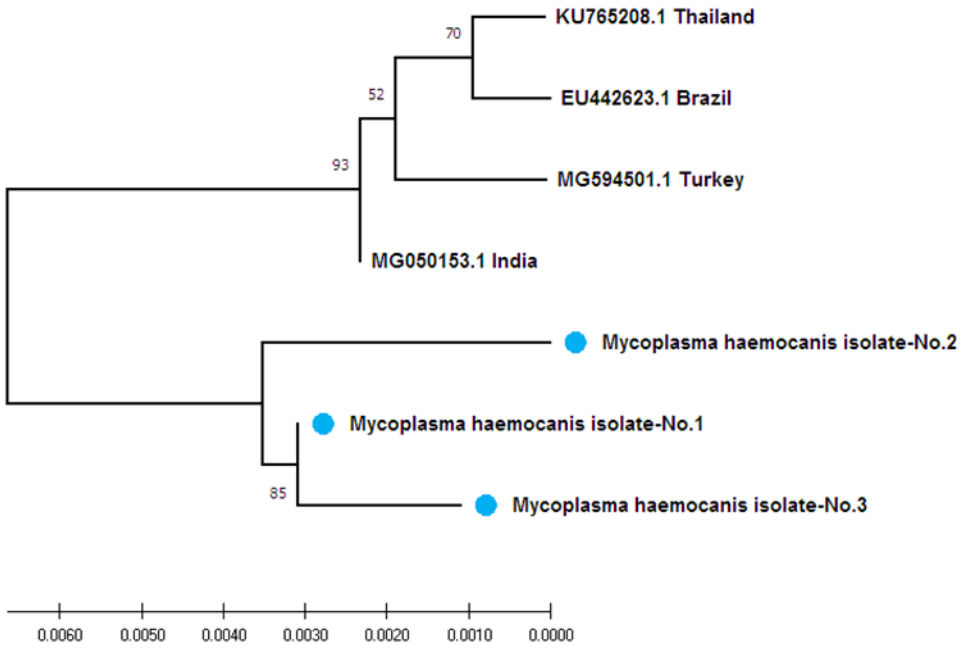

Fig (3): Phylogenetic tree analysis based 16S ribosomal RNAgene partial sequence in local *Mycoplasma haemocanis* dog isolates that used for genetic relationship analysis the phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Mycoplasma haemocanis* dog isolates (No.1- No.3) were showed genetic variant related to NCBI-BLAST *Mycoplasma haemocanis* Turkey, Thailand, Brazil, and India isolates. At total genetic changes (0.0060-0.0010%).

Discussion

Hemomycoplasma characterized by parasitism of the surface of erythrocytes of different mammalian species in which they cause anemia with inconstant severity and from asymptomatic to symptomatic infection result of the present study showed that out of 100 dogs examined, 72% were positive based on direct microscopic examination whereas, only 33 of 72(45.8%) were positive to conventional PCR technique, and blood smears stained from suspected infected dogs showed that *Mycoplasma haemocanis* are small coccoid or rod-shaped structures that can be found as singular or in chains on the RBCs cell membranes of infected animals, and others have reported the same result (17,18,19). Moreover, the high parasitism period may last more than five days; however, the organism may become less frequent since anemia developed (20), also this corresponds to (8,21,22). Other study includ850 dogs in Italy,Spain,and Portugal,and 83(9.6%) were PCR-positive for canine hemoplasma positive dogs (14). may be due to at greater risk of exposure to *R. sanguineus* ticks and fleas (23). The clinical signs that appeared on dogs infected with *M. haemocanis* are variable and non-specific, such as loss of appetite,

lethargy, weight loss, depression, fever, pallor of the mucous membrane, the presence of ticks in suspicious dogs and weakness, and this explained by (11,24,25), other signs were mentioned by (26,19). Whereas the increase in the body temperature of infected dogs reflects the acute characteristic of the disease indicates the release of endogenous pyrogens from the causative agents and because of the cellular degradation that stimulates the centers of thermoregulation in the sub-brain region moreover the severity of the fever may depend on the severity of the causative agents, type of lesion and form of disease, (27,28,29).

Clinical infection with *M. haemocanis* was documented in dogs when identified in Mosul by (30). Present study indicated increased respiratory rate and heart rate may reflect the systemic reaction that occurs due to acute crises of the disease and the pattern of anemia caused by the disease as rapid breathing may affect sick dogs due to anemia hypoxia when a decrease in the of red blood cells and hemoglobin concentration that affected the oxygen that is transported into tissues Therefore, the failure of the tissues to receive an adequate supply of oxygen will occur, and an increase in the respiration of diseased dogs has been clinically revealed by Weiss and (19,31,32)**.**

The presence of pale mucus membranes will exhibit the development of anemia and reduction of blood parameters which was due to destruction and removal of parasitized erythrocytes by the reticulo-endothelial system, whereas icteric mucus membranes which were also seen reflected the progressive anemia (33,34). Hemoplasma is spiny organisms that attach to red blood cells of dogs in some cases, and associated with hemolytic anemia with various severities, ranging from non-clinical hemolysis to severe anemia (35). there are two mechanisms involved in the occurrence of anemia, intra and extra vascular hemolytic, the hemoplasmas induce structural alteration when they bind on the surface of erythrocytes resulting in antigenic modification or exposure of antigens located internally in the cell membrane with consequent production of anti-erythrocyte antibodies by the host. In extra vascular hemolysis there is sequestration and phagocytosis of red blood cells by macrophages of the spleen, liver, lung and bone marrow (4,36) Tasker, 2010 and Hoelzle *et al.*, 2011)..

Current study clarified that there is anemia in infected dogs compared to controls the anemia referred to in the present work is caused by the significant decrease in the values of total erythrocyte count, hemoglobin concentration and packed cell volume the same results have been documented by (3738,39). hemolysis induced by haemomycoplasma infection is usually extravascular and produces regenerative anemia with erythrocyte agglutination. In addition, the increase in mean corpuscle volum shows the appearance of immature red blood cells and is an indicator of regenerative anemia (22,40). The increase in the total leukocyte count WBC indicated in the current study may indicate an increase in the capacity of the immune system increased cellular immunity as leukocytosis can be a reaction to many infectious and inflammatory diseases which is in agreement with (40,38), 2020).

the current study points out ALT and AST values were also increased in diseased dogs. This agree with (22,41,42,43) they reported that damage to the skeleton or cardiac muscle, hepatic

tissue, and red blood cells may lead to a significant increase in AST level, due to the fact that the bulk of that tissue throughout the body can be considered an ample reservoir of enzymes that can be released and detected during a disease (44). In addition it has been documented that increases in ALP activity in the blood are usually due to problems or disease of the liver, biliary and bone, and corticosteroid-induced isoforms, so the elevated ALP activity has been attributed to cholestasis and increased bone activity (24).

Current study, *Mycoplasma haemocanis* was diagnosed with the advent of molecular diagnostic techniques, conventional PCR it is widely used and has known success in both acute and chronic infected animals the same result obtained by other (22,26). This study conforms to roughly the same results with Portugal and the Mediterranean countries, and the highest prevalence using obtained by polymerase chain reaction was in Portugal, of the 50 dogs analyzed, 20 (40%) were positive for *M. haemocanis* (14), in Spain, 26 out of 182 dogs tested positive for *M. haemocanis* and it occur with co-infection with *Candidatus Mycoplasma haematoparvum* and (45) the prevalence of *M. haemocanis* using molecular technique, in France, Spain, Trinidad and Tobago, the United States and Greece was 3.3% (15/460), 14.3% (26/182) ,0.6% (3/506), 5.6% (8/142),respectively (2,7,45,46) respectively in a study of dogs from Italy, Spain and Portugal, the prevalence of *M. haemocanis* (22/600) 3.7%, (1/200) 0.5% and (20/50) 40.0%, respectively (14). *Mycoplasma spp*. also observed its prevalence in Iran at 23% (47).

Multiple sequence alignment analysis of ClustalW alignment analysis based on partial evolutionary distances for the 16SrRNA gene computed using the maximum likelihood method synthesized by UPGMA and local *Mycoplasma haemocanis* dog isolates showed a genetic variant related to NCBI-BLAST *Mycoplasma haemocanis* in Brazil, India, Turkey, Thailand, In total genetic changes (0.0060-0.0010%) this may be due to climate (48).

in conclusion *Mycoplasma haemocanis* have been identified in dogs in Basrah city with various clinical manifestation from in apparent to sever anemia ,emphasized the infection using conventional PCR technique,and Phylogenetic analysis confirm the identification of *Mycoplasma haemocanis* as a new submission of local Iraq, with 99% identical to India, Brazil, Thailand and Turkey DNA sequence.

Acknowledgement

Thank to the officers and associates of the Police Dog Administration and Training Department in the following governorates (Dhi Qar, Basra, Muthanna and Maysan) for their assistance, direction, and support in completing this study**.**

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دراسة سريرية كيموحيوية وجزيئية لل *Mycoplasma haemocanis* في الكلاب في محافظات العراق الجنوبية

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الخلاصة

هدفت الدراسة الحالية إلى الكشف عن عدوى الميكوبلازما الدموية في الكلاب والتعرف عليها من خلال المظاهر السريرية ، و المسحة الدموية ، وتقنية تفاعل البوليميراز المتسلسل التقليدية وتحليل التسلسلات. تم فحص ١٠٠ كلب ، و ٢٥ كلبًا سليمًا سريريا في محافظات جنوب العراق. تم أخذ عينات الدم لتحليل صورة الدم و الاختبارات الكيمياء الحيوية. ٧٢ (٧٢٪) من عينات الدم كانت موجبة للميكوبلازما الدموية، بينما ٣٣ (٤٥,٨٪) إيجابية في فحص تفاعل البلمرة المتسلسل. أظهر الفحص الدموي للحيوانات المصابة انخفاضًا معنويًا في متوسط TRBC و Hb و PCV وزيادة معنوية ($p> 0.05$) في TLC. يشير الفحص البيوكيميائي إلى زيادة معنوية في AST و ALP و ALT في الحيوانات المصابة. اظهرت الميكوبلازما الدموية المحلية تشابه جيني مع عزلات الميكوبلازما الدموية في بنك الجينات NCBI-BLAST لعزالات تركيا وتايلاند والبرازيل والهند. و استنتجت الدراسة الى تشخيص الميكوبلازما الدموية في الكلاب في مدينة البصرة واضهرت علامات سريرية مختلفة الى فقر الدم الحاد، وأكدت الاصابه باستخدام تقنية تفاعل البلمرة المتسلسل، ويؤكد التحليل الوراثي تحديد الميكوبلازما الدموية على أنها عزله عراقيه محليه مع ٩٩ ٪ متطابقة مع تسلسل الحمض النووي في الهند والبرازيل وتايلاند وتركيا.

الكلمات المفتاحية: *Mycoplasma haemocanis*، علامات ، تفاعل البلمرة المتسلسل.