

## Detection of *Anaplasma phagocytophilum* in cows in Mosul city, Iraq

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### Article information

#### Article history:

Received 28 May, 2023  
Accepted 30 July, 2023  
Available online 15 December, 2023

#### Keywords:

*Anaplasma phagocytophilum*  
Microscopic examination  
N-PCR  
Kappa value  
Iraq

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### Abstract

This study was targeted to evaluate the infection rate of *Anaplasma phagocytophilum* in cows in Mosul city-Iraq, using microscopic examination (ME) of the blood smears and nested polymerase chain reaction (N-PCR) technique to evaluate the efficiency of different diagnostic methods used in this study and to determine the parasitemia with the more common leucocytes infected with *A. phagocytophilum*. 50 blood samples were collected from cows in Mosul city. Blood smears were prepared for the primary detection of inclusion bodies (Morulae) inside leukocytes. The remaining blood was used to confirm the infection using the N-PCR technique. Results revealed that the infection rate of *A. phagocytophilum* in Mosul City was 58 and 72% using the ME method and N-PCR, respectively. Substantial compatibility was observed between the ME method and N-PCR technique based on the Kappa value 0.699 with a sensitivity of 80.55%, specificity of 100%, and accuracy of 86% of the ME method compared with the N-PCR technique. Infected cows with *A. phagocytophilum* were suffering from acute disease. Based on the ME of the blood smears, the total parasitemia of *A. phagocytophilum* inside the leukocytes ranged between 6-29%, with a mean of 17%. The highest significance of parasitemia in neutrophils was 40%, followed by lymphocytes was 25% compared with mononuclear cells, basophils, and eosinophils, which showed no significant change of parasitemia among them. In conclusion, *A. phagocytophilum* is widespread in cows in Mosul- Iraq; the ME method and N-PCR technique efficiently detect the *A. phagocytophilum* and neutrophils, which are mostly infected with bacteria.

DOI: [10.33899/ijvs.2023.140648.3074](https://doi.org/10.33899/ijvs.2023.140648.3074), ©Authors, 2024, College of Veterinary Medicine, University of Mosul.  
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### Introduction

*Anaplasma phagocytophilum* represents three species of granulocytic bacteria, including *Ehrlichia phagocytophilum*, *Ehrlichia equi*, and the causative agent of human granulocytic ehrlichiosis (HGE) (1). It is an important pathogen of emerging infectious blood diseases (2,3) and infects different types of animals such as cattle (4), sheep and goats (5,6), horses (7), deer and camels (8,9), dogs and cats (10), rodents and wild mammals (11), and birds (12), as well as human (13). *Anaplasma phagocytophilum* causes tick-borne fever (TBF) or pasture fever disease in ruminants (14,15). It is an obligate intracellular, gram-negative polymorphic bacterium belonging to the order Rickettsiales

of the family Anaplasmataceae and genus *Anaplasma* (16), which is observed in various forms, spherical or spherical rods, or they are polymorphic and form basal colonies in the cytoplasm of different types of white blood cells such as neutrophils, basophils, eosinophils, lymphocytes and monocytes called morulae when examining a blood smear stained with Giemsa stain (14,17). Tick-borne fever disease is mainly transmitted by Ixodidae ticks belonging to the genus of Ixodes, *Hyalomma*, *Rhipicephalus*, and *Boophilus* (17,18). It is also transmitted via blood transfusion from infected animals to healthy animals (19), and bloodsucking flies belong to the Tabanidae family (20). Moreover, Henniger *et al.* (14) mentioned that TBF disease can be transplacental transmission from the infected dam to the

fetus. The disease of *A. phagocytophilum* causes great economic losses as a result of the high mortality, and mortality rates, lack of milk production, stillbirth, and abortion, in addition, to the sums spent to treat infected animals and control the agent that transmits the disease (17, 21). The disease is endemic in about 42 countries, including Asia, Europe, Africa, and America (22-24), and highly morbidity and mortality rates in infected animals (25). The TBF disease appears in various forms, such as acute, chronic, and subclinical (8,26). The most important clinical manifestations of cattle infected with *A. phagocytophilum* include fever 40-41°C, sudden decrease in milk production, inappetence, anemia, stiff gait, lower limb edema, enlargement lymph nodes, stillbirth, and abortion (23,26,27). Clinical signs rarely help in the diagnosis of the disease, as it is associated with other blood diseases that lead to high temperatures, anemia, and decreased milk production in cattle, like Theileriosis (28,29), Babesiosis (30), and Trypanosomiasis (31). Therefore, laboratory tests are usually performed for confirmatory diagnosis of *A. phagocytophilum*, such as microscopic examination of the blood smears stained with Giemsa for detection morulae of *A. phagocytophilum* (27), serological tests like indirect enzyme-linked immunosorbent assay, Indirect fluorescent antibody test (23), and the rapid SNAP 4Dx Plus test for detection highly specific antibodies for *Anaplasma* spp. (32). Furthermore, Molecular techniques such as nested polymerase chain reaction (PCR) techniques (33), quantitative real-time PCR techniques (34), and restriction fragment length polymorphism techniques (6). In Iraq, the *A. phagocytophilum* has been reported in cows in different provinces of Iraq, including Nineveh province using microscopic examination of blood and milk smears (35-37), Al-Qadisiyah, Al-Najaf Al-Ashraf, and Babylon provinces using polymerase chain reaction (PCR) techniques (4,27).

Due to a scarcity of studies on diagnosing *A. phagocytophilum* in cows in Mosul city of Iraq, using recent laboratory methods. Therefore, this study was designed to detection of *A. phagocytophilum* in cows for the first time in Mosul city, Iraq using the ME method and N-PCR technique to evaluate the compatibility and efficiency between these involved methods and to determine the more common leucocytes infected with *A. phagocytophilum*.

## Materials and methods

### Ethical approval

This study was ethically permitted by the animal ethics committee of the College of veterinary medicine, university of Mosul (UD.VET. 2022.031) on 1 August 2022.

### Animals and samples collections

This study was applied to include 50 cows of different sexes, ages 1-5 years, breeds, and management practices, obtained from various regions of Mosul city, which were

clinically suspected infected with *A. phagocytophilum*. During the period from September 2022 to April 2023. Fifty blood samples (3ml of blood) were drawn from all cows via the jugular vein and then kept in tubes with anticoagulant Ethylene Diamine Acetic Acid (EDTA) for preparing blood smears if it is not possible to make from ear vein. Furthermore, the remaining blood was stored at -20°C until used for DNA extraction.

### Microscopic examination of the blood smears

One hundred thin, thick blood smears were prepared from blood samples, stained with ready Giemsa stain (AFCO, Jordan), then examined under the light microscope to detect *A. phagocytophilum* inclusion bodies (Morulae) inside the leukocytes. In addition, the percentage of *A. phagocytophilum* bacteria (Parasitemia) was calculated following the equations (35,36). While the biometrical data of the morulae in various leukocytes were recorded using a digital camera (OMAZ, China), which is fixed on the light microscope.

### DNA extraction and amplification for N-PCR

The DNA was extracted from cow's blood samples (n=50) using the AddPrepGenomic DNA Extraction Kit (Add Bio, Korea). The process was performed as mentioned by the manufacturer. Using the Nanophotometer (BioDrop, Germany), regarding wavelength 260nm, the concentration of extracted DNA was estimated. Additionally, the purity of extracted DNA was calculated by a ratio of (A260 nm to A280 nm).

The N-PCR technique was used to amplify the highly conserved 16S rRNA gene of *A. phagocytophilum*. The DNA extracted from clinically and laboratory-positive cows for *A. phagocytophilum* was used as a positive control. Furthermore, the negative control was performed by adding all PCR competent except DNA. The oligonucleotides of specific primers were designed by Yousefi *et al.* (37). These primers were supplied by (Macrogen Inc. South Korea), which comprising for the first round (1st R.) primer P1 (5'-AGAGTTTGTATCCTGGCTCAG-3'), and primer P2 (5'-AGCACTCATCGTTTACAGCG-3'), and for the second round (2nd R.) primer P4 (5'-GTTAAGCCCTGGTATTTTCAC-3'), and primer P6 (5'-CTTTATAGCT TGCTATAAAGAA-3'). To identify the positive cows for *Anaplasma* spp. Using the primers (P1 and P2), were in approximately band size at 781 bp. in the 1st R., and to identify the positive cows for *Anaplasma phagocytophilum* using the primers (P4 and P6) were in approximately band size at 509 bp. in the 2nd R.

Moreover, the 1st R. and 2nd R. of the N-PCR technique were performed with a total volume of 25µl for each, including (2X) master mix 12.5µl, each primer (P1, and P2), (P4, and P6) 1µl (10 pmol) respectively, template DNA 2µl for 1st R., and PCR product 1µl from 1st R. as template DNA for 2nd R., and complete the total volume with nuclease-free

water 8.5µl, and 9.5µl respectively. The program setting for the thermocycler (BIO-RAD/ USA) for the 1st R. and 2nd R. were as follows: predenaturation step 5min at 95°C (1 cycle), denaturation step 30s at 94°C, annealing step 1min at (58°C for 1st R.), (55°C for 2<sup>nd</sup>R), and extension step 45s at 72°C (35 cycles), with a final extension step 5 min at 72°C (1 cycle), according to Yousefi *et al.* (37) with some steps modification. The final PCR products were loaded in a 1.5% agarose gel stained with Safe-Red™ dye, and the resulting bands were visualized under UV transillumination (BIO-RAD/ USA).

**Comparison between methods used in this study**

The compatibility between the ME and N-PCR techniques was checked based on Kappa values. There was no compatibility between the two tests; if the Kappa value is < 0.00, the compatibility is low; if the Kappa value is ranged 0.0 - 0.20, the compatibility is fair; if the Kappa value is ranged 0.21 - 0.40, the compatibility is moderate; if the Kappa value is ranged 0.41 - 0.60, the compatibility is substantial; if the Kappa value is ranged 0.61 - 0.80, and the compatibility is almost perfect; if the Kappa value is ranged 0.81 - 1 (38). Moreover, the ME method's accuracy, sensitivity, and specificity were computed and compared to the N-PCR technique (39).

**Statistical analysis**

X<sup>2</sup>- test and Kappa value were used by IBM-SPSS Version 22 (Inc., Chicago, USA) to analyze the data in this study. Statistically significant data was determined at the P<0.05.

**Results**

The current work revealed that the infection rate of *A. phagocytophilum* in Mosul City was 58% (29/50) using ME of the blood smears and 72% (36/50) using N-PCR (Table 1). Based on the Kappa value (0.69), substantial compatibility was observed between the ME method and the N-PCR technique with a sensitivity of 80.55%, specificity of 100%, and accuracy of 86% of the ME method compared with the N-PCR technique (Table 2). This indicates that the ME method and N-PCR technique are suitable for detecting *A. phagocytophilum* in animals.

The results of the study also revealed that the infected cows (n=36) with *A. phagocytophilum* suffered from the acute form of the disease with loss of appetite, general weakness, fever (40.5-41°C), enlargement of the superficial lymph nodes, and pallor of the mucous membranes of the eyes, as well as respiratory, and cardiac disorders. Some of the infected cows also showed edema of the limbs, diarrhea, dehydration, staggering gait, recumbency, decreased milk production, and ticks on different body parts, with different frequencies and percentages (Figure 1).

Table 1: Infection rate of *Anaplasma phagocytophilum* in cows at Mosul city using microscopic examination (ME) method, and N-PCR technique

Type of test	No. of tested cows	No. of positive cows	Infection rate %
ME method	50	29	58
N-PCR technique		36	72

Table 2: Compatibility between microscopic examination, and N-PCR technique based on kappa value, calculating the ratio of the ICA rapid test's sensitivity, specificity, and accuracy for FPV diagnosis

Microscopic examination	Nested PCR technique		
	Infected	Uninfected	Total No.
Infected	29 a	0 b	29
Uninfected	7 c	14 d	21
Total	36	14	50

(a) True positive samples, (b) False positive samples, (c) False negative samples, (d) True negative samples. Kappa value was 0.699. Sensitivity were equal to a/(a+c)\*100= 80.55%. Specificity were equal to d/(b+d)\*100= 100%. Accuracy were equal to (a+d)/(a+c+b+d)\*100= 86%.

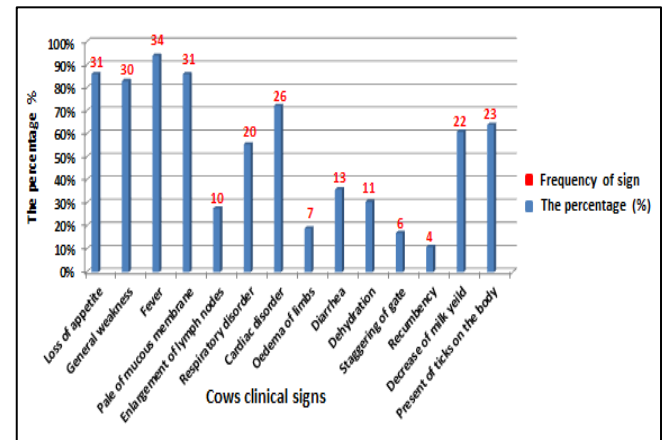


Figure 1: Frequency, and percentage of clinical signs shown on cows infected with *Anaplasma phagocytophilum* (n= 36).

Based on ME of 100 thin and thick blood smears stained with ready Giemsa stain, the inclusion bodies (Morulae) of *A. phagocytophilum* appear in all types of white blood cells, often individually or more than one Morulae inside the cytoplasm of the same cell. The length and width of Morulae ranged between 1.45 µm - 10.45 µm, with the mean 4.05 µm, and the between 0.95 µm - 5.84 µm, with the mean 2.86 µm respectively (Figure 2), with the total parasitemia of *A. phagocytophilum* Morulae inside the leukocytes was ranged between 6-29% with the mean 17%. The highest significance (P<0.05) of *A. phagocytophilum* parasitemia was observed

in neutrophils 40%, followed by lymphocytes 25% compared with mononuclear cells, basophils, and eosinophils, which showed no significant change in the parasitemia among them, this indicates that neutrophils were most commonly leukocytes infected with *A. phagocytophilum* (Table 3). The extracted DNA's concentration and purity ranged between 80.9 - 370.5 ng/  $\mu$ l and 1.7 - 1.9, respectively. Furthermore, based on the N-PCR technique for amplified DNA fragments of the 16S rRNA gene of *A. phagocytophilum* in 50 blood samples from cows observed in the 1st R. the positive cows for Anaplasma spp., in approximately band size at 781 bp (Figure 3), and in the 2nd R., the positive cows for *A. phagocytophilum* in approximately band size at 509 bp (Figure 4).

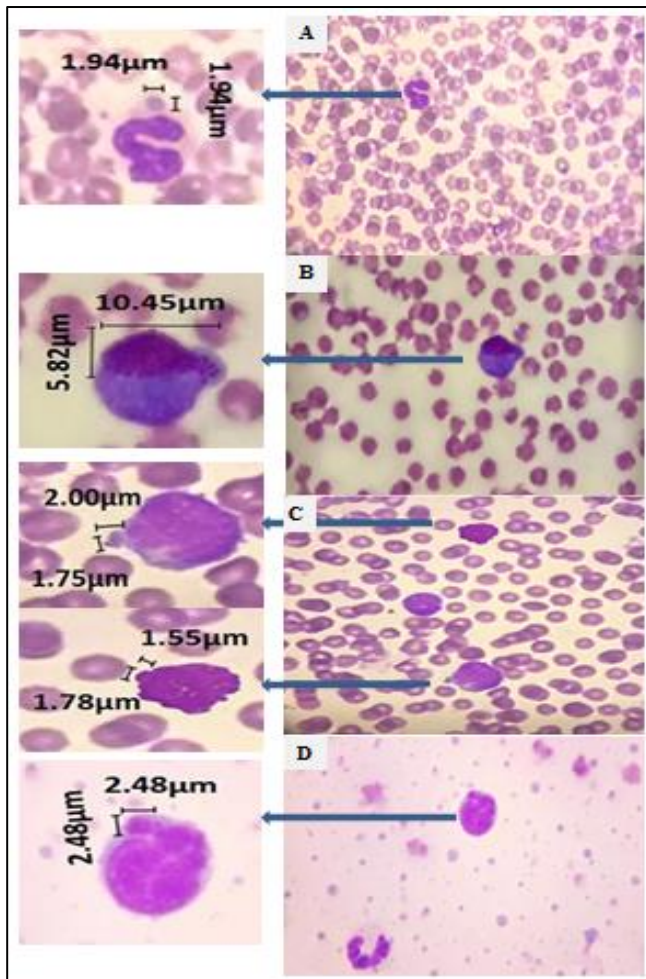


Figure 2: Blood smears stained with Ready Giemsa stain showed inclusion bodies (Morulae) of *A. phagocytophilum*: A- Inside cow neutrophil; B- Inside cow basophil; C- Inside cow eosinophil and lymphocytes; D- Inside cow monocyte, examined using a light microscope under oil immersion at (1000X).

Table 3: Parasitemia of *A. phagocytophilum* (Morulae) in total and different white blood cells in blood smears of infected cows.

Type of cells	Range% (Mean $\pm$ Standard Error)
Leukocytes	6-29% (17 $\pm$ 1.21)
Neutrophils	10-70% (40 $\pm$ 3.24 a)
Lymphocytes	5-60% (25 $\pm$ 2.49 b)
Monocytes	5-15% (8 $\pm$ 0.59 b)
Basophils	0-5% (5 $\pm$ 0.49 b)
Eosinophils	0-10% (5 $\pm$ 0.43 b)

A significantly different ( $P < 0.05$ ) was assigned by different superscript letters (a,b).

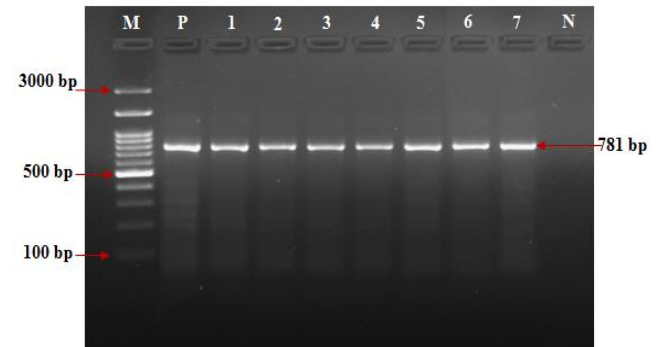


Figure 3: Representing the 1st R. of N-PCR: lane M: Exact Mark 100-3000bp DNA ladder; Lane P) DNA extracted from infected cow used as a positive control for *A. phagocytophilum*; Lanes 1-7) The positive cows for Anaplasma spp. in approximately band size 781bp.; Lane N) add all PCR competent except DNA used as the negative control.

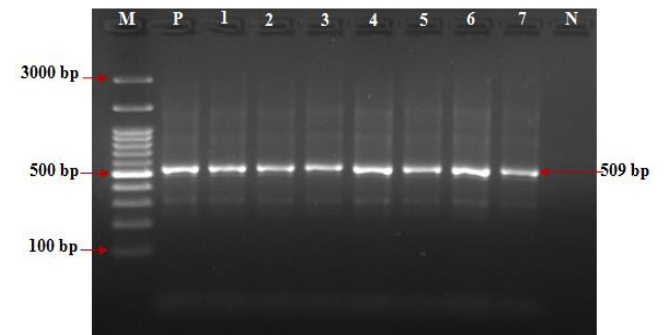


Figure 4: Representing the 2nd R. of N-PCR: lane M: Exact Mark 100-3000bp DNA ladder; Lane P) DNA extracted from infected cow used as a positive control for *A. phagocytophilum*; Lanes 1-7) The positive cows for *A. phagocytophilum* in approximately band size 509 bp.; Lane N) add all PCR competent except DNA used as a negative control.

## Discussion

In the current work, the infection rate of *A. phagocytophilum* in Mosul City was 58% and 72% using the ME of the blood smears and N-PCR technique. These results are higher than the other studies that reported *A. phagocytophilum* in Iraq. Ayyez *et al.* (27) stated that the infection rate of *A. phagocytophilum* in Al-Qadisiyah province was 40% using conventional PCR techniques, respectively. Also, the infection rate in Al-Najaf Al-Ashraf and Babylon provinces was 6.15% and 4.61% using N-PCR techniques (4). Furthermore, there are various studies around the world observed varying in the infection rate of *A. phagocytophilum* in cows using different laboratory techniques in different provinces of Iran was 15.5% using the N-PCR technique (24), in Turkey was 30.8% using reverse line blot hybridization assay (22), in South Korea, and Ethiopia was 2.1%, and 2.73% using restriction fragment length polymorphism technique (40,41), in Egypt was 5.3% using Real-time PCR technique (42), in Tunisia was 0.6% using Duplex-PCR technique (43), and in Northern Germany was 60% using ME of blood smears (26). The variations in the infection rate of *A. phagocytophilum* in a variety of regions and countries have belonged to different management practices, environmental conditions, efficient diagnostics techniques used in various studies, sample size, and presence and/or absence of many factors such as age, immune status of host, and the presence of ticks in the field, and on animals (24,44,45).

This study indicates substantial compatibility observed between ME methods and the N-PCR technique based on the Kappa value, with higher sensitivity, specificity, and accuracy of the ME method compared with the N-PCR technique. This indicates that the ME method and N-PCR technique are suitable for detecting *A. phagocytophilum* in animals. These findings were consistent with Noaman, Shayan (46), and Nider *et al.* (47), who mentioned that ME of blood smears, and PCR technique are efficient for diagnosing *A. phagocytophilum*. On the other hand, Silaghi *et al.* (23) and Ola-Fadunsin *et al.* (48) stated that ME of blood smears is an easy, rapid, and cheap field test that can be used in the diagnosis of acute cases of *A. phagocytophilum* in cows, but this method has low a sensitivity, and rarely yield positive results in cases of chronic, subclinical, or persistent infection, as in these conditions the percentage of parasitism is very low. Therefore, the results of the ME method should be confirmed using more sensitive and accurate techniques such as serological testing and molecular techniques.

In this study, cows infected with tick-born fever (TBF) disease were suffering from the acute form of the disease with different clinical manifestations, including loss of appetite, general weakness, fever, enlargement of the lymph nodes, pallor of the mucous membranes, as well as respiratory, and cardiac disorders, edema of the limbs,

diarrhea, dehydration, staggering of gait, recumbency, decrease of milk production, and presence of ticks on different parts of the body. This result is in congruence with Constable *et al.* (8), Norman (24), and Ayyez *et al.* (27). The tick-born fever (TBF) disease causes immunosuppression of infected cows, leading to secondary infections such as tick pyemia due to *Staphylococcus aureus*, pneumonia due to *Pasteurella multocida*, septicemic Listeriosis, and encephalitis due to louping-ill virus, and secondary infection due to opportunistic fungi (17,49,50).

Results based on ME of staining blood smears demonstrated the inclusion bodies (Morulae) of *A. phagocytophilum* inside all types of white blood cells, often singly or more than one in the cells, with the Morulae range of the length (1.45  $\mu\text{m}$  - 10.45 $\mu\text{m}$ ), and width (0.95  $\mu\text{m}$  - 5.84  $\mu\text{m}$ ). This finding agrees with that observed by Atif (21) and Taylor *et al.* (16). Furthermore, the total parasitemia of *A. phagocytophilum* (Morulae) inside the leukocytes was ranged between 6-29% with the mean 17%, which consistent with Al-Badrani, (35) mentioned that the parasitemia of *A. phagocytophilum* ranged between 1-25% in the blood smears prepared from cows' blood. Additionally, results indicate that neutrophils were most commonly leukocytes infected with *A. phagocytophilum*; this result agrees with the finding of Ebani *et al.* (51), Taylor *et al.* (16), and Rassouli *et al.* (9), who refer to *A. phagocytophilum* is infected all leukocytes with a specialty in the neutrophils.

## Conclusions

This study states that *A. phagocytophilum* is widespread in cows in Mosul- Iraq; with the acute form of the disease, the ME method and N-PCR technique are efficient for the detection of *A. phagocytophilum*. Neutrophils are the most commonly leukocytes infected with bacteria.

## Acknowledgments

We want to express our deepest gratitude to the College of veterinary medicine, university of Mosul, Nineveh, Iraq, for their support in conducting our study.

## Conflict of Interest

No conflicts of interest exist, according to the authors, with the publishing of this work.

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## الكشف عن الانابلازما فاكوسيتوفيلوم في الأبقار في مدينة الموصل، العراق

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### الخلاصة

هدفت الدراسة الحالية إلى تقييم معدل الإصابة بجراثيم الانابلازما فاكوسيتوفيلوم في الأبقار في مدينة الموصل-العراق، باستخدام الفحص المجهرى للمسحات الدموية وتقنية تفاعل البلمرة المتسلسل المتداخل، وتقييم كفاءة الطرائق التشخيصية المختلفة المستخدمة في هذه الدراسة، مع تحديد نسبة التطفل ونوع خلايا الدم البيضاء الأكثر إصابة بجراثيم الانابلازما فاكوسيتوفيلوم. تم جمع ٥٠ عينة دم (٣ مل) من أبقار مدينة الموصل، مع تحضير مسحات الدم للكشف الابتدائي عن الأجسام الاشتمالية (الموربولا) داخل خلايا الدم البيضاء وتم استخدام باقي الدم لتأكيد الإصابة باستخدام تقنية تفاعل البلمرة المتسلسل المتداخل. أظهرت النتائج أن نسبة الإصابة بجراثيم الانابلازما فاكوسيتوفيلوم في مدينة الموصل بلغت ٥٨ و ٧٢٪ باستخدام طريقة الفحص المجهرى وتقنية تفاعل البلمرة المتسلسل المتداخل على التوالي. لوحظ توافق حقيقي بين طريقة الفحص المجهرى وتقنية تفاعل البلمرة المتسلسل المتداخل، اعتمادا على قيمة كابا ٠,٦٩٩ مع حساسية ٨٠,٥٥٪ ونوعية ١٠٠٪ ودقة ٨٦٪ لطريقة الفحص المجهرى مقارنة بتقنية تفاعل البلمرة المتسلسل المتداخل، كما عانت الأبقار المصابة بجراثيم الانابلازما فاكوسيتوفيلوم من الشكل الحاد من مرض. واعتمادا على الفحص المجهرى للمسحات الدموية تراوح إجمالي نسبة التطفل لجراثيم الانابلازما فاكوسيتوفيلوم داخل خلايا الدم البيضاء بين ٦-٢٩٪ بمعدل ١٧٪، وكانت أعلى نسبة تطفل معنوية في الخلايا العذلة بنسبة ٤٠٪، تليها الخلايا اللمفية بنسبة ٢٥٪ مقارنة بالخلايا وحيدة النواة والخلايا القاعدية والحامضية والتي لم تظهر أي تغير معنوي في نسبة التطفل فيما بينها. استنتج من هذه الدراسة، أن جراثيم الانابلازما فاكوسيتوفيلوم واسعة الانتشار في الأبقار في مدينة الموصل-العراق، وأن طريقة الفحص المجهرى وتقنية تفاعل البلمرة المتسلسل المتداخل فعالة في الكشف عن جراثيم الانابلازما فاكوسيتوفيلوم، كما تعد خلايا العدلات الأكثر إصابة بتلك الجراثيم.