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Prevalence of hemoplasmosis in sheep in Mosul city, Iraq

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Abstract

Hemoplasmosis is a worldwide disease that infects sheep. It is induced by hemotropic mycoplasma, a widespread zoonotic disease that can result in mild to severe destruction of erythrocytes, jaundice, and inadequate mass growth in mammals. This study aimed to evaluate the effectiveness of conventional polymerase chain (c-PCR) in comparison to microscopic examination (ME) of blood smears stained with the RAL 555 rapid stain kit and tick samples from areas located in Mosul City, Iraq, for estimating the distribution of ovine hemoplasma (OH) in sheep. In the period between July 2024 and January 2025, three hundred sheep were selected from different areas of Mosul to have their blood samples taken. The total number of cases of OH was 79.6% (239 of 300) on microscopic examination and 80.3% (241 of 300) using c-PCR. The infestation rate of hard ticks on sheep was 66.6%, and two species of hard ticks, *Rhipicephalus turanicus* and *Rh. sanguineus*, were microscopically identified and classified, with *Rhipicephalus turanicus* being significantly more common. The concordance between the blood smear examination by microscopy and the c-PCR methodology is nearly flawless, as indicated by a Kappa value of 0.979. The c-PCR approach demonstrates great sensitivity, specificity, and accuracy at 99.1, 100, and 96.3%, respectively, in comparison to the ME method. It was concluded from this study that sheep hemoplasmosis is prevalent in sheep in Mosul, Iraq, diagnosed in blood and ticks parasitizing sheep, and that conventional PCR and microscopy are more effective methods for detecting sheep infected with hemoplasmosis.

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Introduction

Mycoplasma ovis, occasionally referred to as hemotropic mycoplasma (formerly named *Eperythrozoon ovis*), is an uncultivated, pleiotropic bacterium that invades red blood cells. It looks like small, bluish cocci, rings, and spheres that aggregate on the outermost layer of red blood cells (1). It is also found freely in the plasma of some mammalian species. It differs from most mycoplasmas due to the absence of a cell wall, making it susceptible to tetracyclines. Hemolytic anemia in animals can result from infections, but the absence of suitable diagnostic techniques has hindered veterinary research. Despite its low diagnostic sensitivity and inability to differentiate between species, cytological confirmation of microorganisms on blood smears was the method most commonly utilized in research (2-4). Furthermore, given that hemoplasmas and Howell-Jolly bodies frequently arise post-splenectomy, are linked to anemia, and possess DNA, this diagnostic method may erroneously classify hemoplasmas as the latter (5). The hosts are susceptible to asymptomatic infections from this microbe; however, sheep have been shown to exhibit a high temperature, yellowing of the skin, hemoglobinuria, decreased weight, and, occasionally, hemolytic anemia, which may become fatal (5). Hematophagous arthropods may participate in the natural transport of blood plasma, although this is unknown, such *Rhipicephalus sanguineus* (2). According to Sykes *et al.* (6), the extent of the cases is influenced by the host's age, dietary and immune condition, and the existence of simultaneous diseases such as *Anaplasma ovis*. Following the first

discovery of *Mycoplasma ovis* in South African sheep blood samples, other outbreaks were recorded in various parts of the globe. These include Germany (5), Argentina (7), Hungary (8), Australia (9), Malaysia (10), Tunisia (11), and most recently, Turkey (12). During the recent ten years, hemoplasmas species in sheep have emerged as a significant pathogen for human public health due to numerous reported cases involving staff members, including clinicians and wildlife leaders, who have been exposed to animal contact (3,6). As for Iraq, there were three studies in Mosul, southern and central Iraq, Basra Governorate, and the city of Diwaniyah (13-15).

As for northern Iraq, specifically the city of Mosul, there are no studies on this subject. Consequently, this study set out to compare the efficacy of c-PCR and microscopic examination in diagnosing ovine hemoplasmosis in sheep in Mosul, Iraq, as well as to determine the parasite pattern and type.

Materials and methods

Ethical approval

The University of Mosul College of Veterinary Medicine's Institutional Animal Care and Use Committee granted ethical clearance for this research project on July 9, 2024 (UM.VET.2024.04).

Animal and sample dimensions

The number of samples required for the research investigation was established using an epidemiological statistical equation, following the methodology outlined by Charan and Biswas (16), as the researcher estimated the frequency of hemotropic mycoplasma in sheep in Mosul at 17.5%, which is the percentage of a recent study conducted in Al-Qadisiyah Governorate, Iraq (15). The confidence level was 95%, and the standard error rate was 5%. The calculation was made according to the following equation: $n=[(z^2p)*(1-p)]/d^2$. Where (n) is the number of animals sampled, (Z) is the average distribution value at a 95% confidence level, (P) is the expected prevalence, and (d) is the absolute error. Since the sample size calculated according to the equation was 260 sheep, 300 were collected in this study.

Samples collection

A number of 300 blood samples (300 sheep) measuring 2.5 ml each were collected using a 3 ml sterile syringe from sheep's jugular vein, and this was done from July 2024 to January 2025. The samples were later placed in tubes containing the anticoagulant EDTA for storage and transportation. We collected sheep infected with hemoplasma for blood smear testing; each animal had two stains. We froze the remaining blood at -20 degrees Celsius to facilitate its molecular analysis using c-PCR. (17-21). In addition, various sections of the body were used to capture male and female hard ticks (Ixodid ticks), which were then kept in formalin (10%) until microscopic analysis. To identify and detect hemoplasma within other engorged female ticks using the cPCR method, they were maintained in 70% ethanol at 4°C until microscopic examination (22).

Blood smears examination

A total of 300 smears had been made, given time to dry naturally, stained using the RAL 555 quick stain kit (CellaVision, France), and then inspected using an immersion oil light microscope set at X1000 (Leitz, Germany) (23). To conduct initial investigations on RBC hemoplasma species.

Identification and Classification of ticks

Hard ticks (n=100) were recognized and categorized at species and genus levels through physical features using stereo imaging and established taxonomy standards (24-27).

Extraction of DNA

300 sheep whole blood and 50 engorged female tick samples underwent genomic DNA extraction using the manufacturer's instructions using the AddPrep Genomic DNA Extraction Kit from tissue, blood, and plant (Add Bio, Korea). At 260 nm, Nanodrop (BioDrop, England) measured extracted DNA concentrations ranging from 37.6 to 322.7 ng/µL. A proportion of A260 nm to A280 nm calculation revealed an accuracy range of 1.5 to 1.9. (28-30).

Amplification of DNA

The following procedures were employed to enhance the hemoplasma spp. 16S rRNA gene: Using universal primers (H16S-F:5' ATACGGCCCATATTCCTACG 3' and H16S-R:5' TGCTCCACCACTTGTTCA 3'), a c-PCR reaction, with the size of the band ranging from 595 to 620 base pairs, was employed to detect hemoplasma species in sheep that tested positive. Macrogen Inc. of South Korea supplied them all (31). The conventional PCR method required a twenty-five microliters (µL) volume, containing twelve and a half µl of 2X AddBio Master Mix, one µL of all primer (H16S-F and H16S-R) (ten pmol), two µL of DNA (one hundred fifty ng/µL), and 8.5 µL of PCR-grade water. Moreover, the control group that did not receive template DNA but had all other components was also included. As described below, the thermocycler (BIO-RAD/USA) was set up. According to Hampel *et al.* (14), The polymerase activation phase lasts for 10 minutes at 95 degrees Celsius. This is followed by a denaturation stage that lasts for 45 seconds at 95°C, an annealing stage that lasts for 45 seconds at 55°C, and an extension step that lasts for 1 minute at 72°C. This is repeated for 35 moments, with the last extension phase of five minutes at 72°C for 1 cycle. The outputs of amplification have been separated using 1.5% agarose (AddBio, Korea) and 3 µl of GelRed dye. Five microliters of each PCR product were added to the agarose gel. The electrophoresis was carried out at 75 V for one hour using a 300-mA power supply and a

genotyping container (Bio-Rad, USA) with a cycle TBE buffer (GeNetBio, Korea). The 100 base-pair DNA marker (six µL), which came from GeneDirex H3 in Korea, functioned similarly to the usual molecular mass biomarker.

Comparing the various methods employed in this work

The researcher assessed the reliability of the c-PCR method in comparison to the ME of blood smears by calculating the kappa value. Kappa values below zero indicate that there is no agreement between the two tests. Limited confidence is indicated by a Kappa value between 0.0 and 0.20, reasonable concurrence by 0.21 to 0.40, considerable acceptance by 0.41 to 0.60, huge cooperation from 0.61 to 0.80, and nearly ideal confidence by 0.81-1 (13). Additionally, ME sensitivity, specificity, and accuracy were computed using the c-PCR method (32).

Statistical analysis

Information from the present research was examined utilizing International Business Machines-Statistical Product and Service Solutions (IBM-SPSS) Edition 19, obtainable from Inc. in Chicago, USA. The software provided tools such as the chi-square statistic two-by-two table and the Kappa value. The data was considered statistically significant when the P value reached 0.05.

Results

The current study revealed a total frequency of OH in sheep in Mosul of 79.6% (239 from 300) via microscopic evaluation of blood smears and 80.3% (241 from 300) using the conventional PCR approach (Table 1). Infected animals' red blood cells and free plasma contain hemoplasma spp., which was observed in a microscopic analysis of 300 blood smears stained with the RAL 555 quick dye kit. The microorganisms' morphologies range from coccoid to spherical (Figure 1). In addition, the outcomes were achieved by employing conventional polymerase chain reaction on magnified DNA segments derived from hemoplasma species' 16S rRNA genes. Mosul city was the first to examine 300 sheep blood samples with universal catch-all primers. The results showed a positive band at around 595 base pairs (Figure 2).

Table 1: Microscopic analysis and conventional PCR methods have been employed to ascertain the general frequency of hemoplasmosis in the sheep population of Mosul

Used the test	Exanimated sample count	Positive numbers (%)
Microscopic examination	300	239 (79.6)
c-PCR technique		241(80.3)

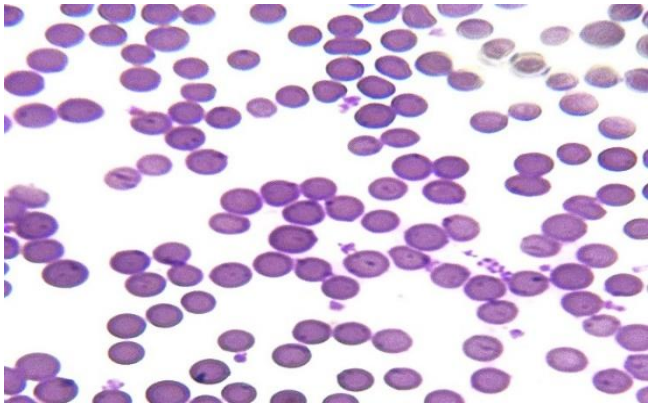

Figure 1: A blood smear with RAL 555 rapid stain revealed hemoplasma parasites in sheep attached to unbound plasma and RBCs, individually or in chains, with changes in the shape and size of red blood cells immersed in oil (100X).

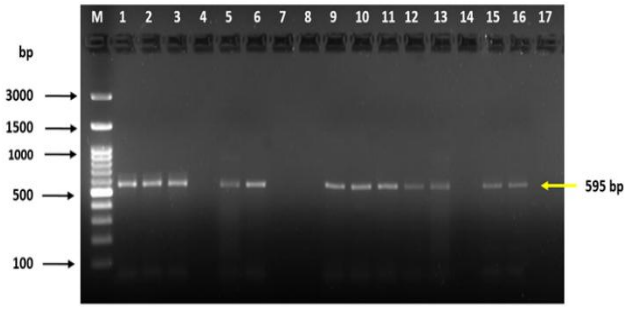

Figure 2: *Mycoplasma spp.* 16SrRNA gene polymerase chain reaction (PCR) utilizing H16S primer from sheep blood samples. Lane M: DNA ladder of 100 base pairs. Lanes 1,2,3,5,6,9-13, 15 and 16 are positive samples. Lanes 4, 7, 8, and 14 are negative samples. Lane 17 is negative control.

The study observed that 66.6% of sheep were infested with hard ticks (200 out of 300). It was determined that there are two distinct species of hard ticks: *Rhipicephalus turanicus* 60% and *Rh. sanguineus* 40%. *Rhipicephalus turanicus* was significantly more prevalent than Rh. sanguineus ($P<0.05$) (Figures 3 and 4). Furthermore, the c-PCR procedure was used to amplify DNA pieces of the small subunit ribosomal RNA (16S rRNA) gene associated with hemoplasma in 50 bloated female parasitic ticks. The results demonstrated that the ticks tested positive for hemoplasma with a band size of around 595 bp (Figure 5 and Table 2).

Regarding the diagnosis of hemoplasma spp. in sheep, the current study also discovered the nearly perfect correlation between the microscopic examination of staining blood smears and the c-PCR methodology, indicated by a Kappa value of 0.979. In comparison to the c-PCR method, microscopic analysis achieved a sensitivity of 99.1%, specificity of 100%, and accuracy of 96.3% (Table 3).

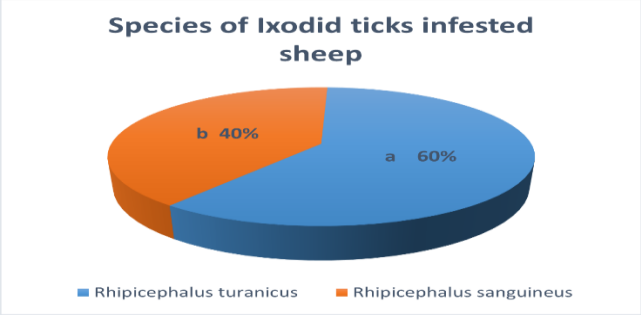

Figure 3: Infested rate of infected and type of hard ticks on sheep (the number = 100) Variables that demonstrated significant changes (P<0.05) were denoted by several superscript symbols (a, b).

Figure 4: Dorsal and ventral views of *Rhipicephalus sanguineus*; ventral image of *Rhipicephalus turanicus*.

Table 2 illustrates the ovine hemoplasma infestation rate in fulminant female Ixodid ticks as determined by the c-PCR method

Species of tick	Engorged female tick's number	Positive number (%)	Microbe %
Rhipicephalus turanicus	25	[a] 20 (80)	*Ovine hemoplasma*
Rhipicephalus sanguineus	25	[a]23 (92)	
Total	50	43	86%

Significant differences in values below the probability level of P<0.05 are indicated by vertical letter differences (a, b).

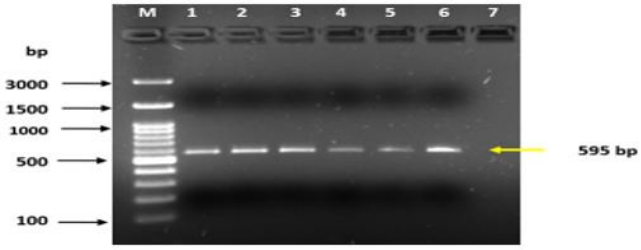

Figure 5: Amplification of the 16SrRNA gene of Mycoplasma spp. from ticks was conducted using the H16S primer through polymerase chain reaction (PCR). Lane M: one hundred base-pairs DNA ladder. Lanes from 1 to 6 are positive samples. Lane 7 is negative control.

Table 3 provides a comparison of the kappa values of conventional PCR (c-PCR) and microscopic examination (ME), as well as ME's precision, sensibility, and reliability in diagnosing OH.

Microscopy examination	The PCR method		
	Infected	Healthy	Overall Quantity
Infected	239 a	0 b	239
Un-infected	2 c	59 d	61
Total	241	59	300

(a) true positive samples, (b) false positive samples, (c) false negative samples, (d) true negative samples. Kappa value was 0.979. Sensitivity = a/(a+c)*100= 99.1%. Specificity = d/(b+d)*100 = 100%. Accuracy = (a+d)/(a+c+b+d)*100= 96.3%.

Discussion

The current study found that c-PCR detected 80.3% of OH cases and ME blood smears 79.7% in sheep in Mosul. Prior research on OH in Iraq found a lower prevalence than these results. Hasan (13) found that 40% of sheep in Mosul, Iraq, had the disease, employing ME of blood-stained smears. In Al-Diwaniyah, Iraq, Kshash (15) used c-PCR to find a frequency of 17.5% in sheep. In Basrah, South Iraq, Abed and Alsaad (14) found that the prevalence of OH was 100%, according to the stained blood smears examined under a microscope and ELISA. Differences through the distribution of OH in sheep across various geographic areas within the exact same nation can potentially be attributed to breeding practices, testing methodologies, the availability of tick carriers in the environment or on the livestock, the quantity of the specimen obtained, and atmospheric factors such as seasons that influence the tick population (29).

As with other livestock HM species, additional worldwide research has shown that different experimental approaches reveal different amounts of hemoplasma spp prevalence in sheep (30-34). Examples include the USA 69-79% (35), Brazil 79% (31), Hungary 52% (8), Turkey 54% (12), Tunisia 6% (11), the Philippines 36% (36), and China 45% (37). Hemoplasma species prevalence may vary from country to country due to factors such as management methods, diagnostic efficiencies, tick prevention strategy efficacy, the existence of effective tick vectors, and ecology (25-37). The Kappa value of 0.979 indicates an almost perfect concordance between the analysis of c-PCR results

and blood smears; nevertheless, the ME method exhibits superior reliability, specificity, and accuracy relative to c-PCR. This discovery corresponds to the findings of Hampel (30). Hemoplasma can probably be seen on erythrocytes under a microscope while an acute case of OH is being investigated. Microscopic inspection is convenient, quick, cheap, and easy to use, but it should not be used in place of more delicate and accurate methods like molecular and serology testing (38). Because of its accuracy and the capability to examine DNA from 2.5 µl of blood, the PCR test is frequently employed to identify DNA from diseased animal hemoplasma (39).

This study found that two types of hard ticks have been recognized and studied microscopically and that 66.6% of sheep were infested with these insects: *Rhipicephalus turanicus* and *Rh. sanguineus*. This finding is consistent with previous research, which has found these Ixodid tick species throughout different Iraqi provinces (40-42). Furthermore, in the current study, Rhipicephalus turanicus was substantially more frequent than Rh. sanguineus, which could be related to its capacity to resist the arid and severe climate. The conclusion was similar to prior investigations (43-45). In addition, the results also corroborated earlier research showing that c-PCR can be used to diagnose hemoplasma infection in DNA collected from filled female ticks (46,47).

Conclusions

The c-PCR approach has demonstrated that OH is frequent in Mosul, Iraq, and, together with microscopic testing, is the most effective and dependable tool for evaluating whether an animal is infected with hemoplasma. The main carriers of these bacteria are *Rhipicephalus turanicus* and *Rhipicephalus sanguineus*. A carefully constructed tick prevention strategy should be implemented to inhibit the spreading of this microbe.

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Conflict of interest

This paper is free of any conflicts of interest, as the authors attest.

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انتشار مرض الهيموبلازما في الأغنام في مدينة الموصل، العراق

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الخلاصة

داء الهيموبلازما هو مرض عالمي يصيب الأغنام. وهو ناتج عن الميكوبلازما الدمية، وهي مسببات أمراض حيوانية واسعة النطاق يمكن أن تسبب فقر الدم الانحلالي الخفيف إلى الشديد، واليرقان، وضعف اكتساب الوزن لدى الحيوانات. هدفت الدراسة الحالية تحديد مدى انتشار مرض الهيموبلازموسس في الأغنام باستخدام الفحص المجهري للمسح الدموية المصبوغة بالصبغة السريعة وتفاعل البلمرة المتسلسل التقليدي وعينات القراد في مدينة الموصل- العراق، وكما تم المقارنة بين تقنية تفاعل البلمرة المتسلسل التقليدي والفحص المجهري كتقنيات لتشخيص المرض، في الفترة بين تموز ٢٠٢٤ إلى كانون الثاني ٢٠٢٥، تم سحب ٣٠٠ عينة دم من الأغنام في مناطق مختلفة من مدينة الموصل. وبلغت نسبة الانتشار الكلي للمرض في الضأن) ٧٩,٦٪ (٢٣٩ من أصل ٣٠٠) و ٨٠,٣ ٪ (٢٤١ من أصل ٣٠٠) باستخدام الفحص المجهري وتقنية تقنية تفاعل البلمرة المتسلسل التقليدي على التوالي. بلغ معدل الإصابة بالقراد الصلب في الأغنام ٦٦,٦%، وتم التعرف على نوعين من القراد

الصلب وهما القراد البني الصلب وقراد الكلب البني وتصنيفهما مجهريا، وكان القراد البني أكثر شيوعا بشكل ملحوظ. لوحظ توافق حقيقيا بين الفحص المجهري للمسحات الدم وتقنية تفاعل البلمرة المتسلسل التقليدي اعتمادا على قيمة كابا التي بلغت ٠,٩٧٩، مع ارتفاع في حساسية ونوعية ودقة طريقة تفاعل البلمرة المتسلسل والتي كانت٩٩,١%, ١٠٠%, ٩٦,٣% على التوالي مقارنة بتقنية الفحص المجهري. استنتج من هذه الدراسة إلى أن داء الهيموبلازما في الأغنام منتشر في الأغنام في الموصل بالعراق، والذي تم تشخيصه في الدم والقراد المتطفل على الأغنام، وأن تفاعل البلمرة المتسلسل التقليدي والفحص المجهري هي طرق أكثر فعالية للكشف عن الأغنام المصابة بالهيموبلازما.