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Diagnostic Study of *Theileria Annulata* Parasite in Cows in Dhi Qar Governorate

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Abstract

This study was conducted in Dhi Qar Governorate / Iraq to diagnose cows being infected with *Theileria annulata*, which is a protozoan parasite that infects cows. 214 cattle blood samples were collected from different areas of the governorate that were diagnosed with a microscope through blood smears stained with Gemza stain, where the annular phase of the pyroplasm was seen positive. By (19%) (41/214) using a light microscope, while when using the polymerase chain reaction technique(PCR), (50%) was diagnosed with (50/100) positive, as this technique is considered more accurate in detecting parasites

Key words: PCR, Theileria annulata , cattle

Introduction

Theileriosis is a parasitic disease caused by the parasite Theleria.sp. It is considered one of the hematopoietic precursors that infect erythrocytes and lymphocytes in ruminants spread all over the world, where the disease is transmitted by Ixodid ticks that infect ruminants (Ayadi et al., 2016). Negatively affects livestock, causing large economic losses The Theileria parasite belongs to the Apicomplexa phylum, Sporozoea class, Proplasmida, the Theileriidae family (Knowles el at. 2018). The form of the thalliaria parasite is small, round, oval, irregular, or bacillary, with the apical shape containing rhoptries. Classification of the subspecies of the thallaria parasite is based on the form of the parasite, the host type, the clinical signs of the host and the tick vector, and the geographical area (jalovecka et al., 2019). The diagnosis of theileria parasite is based on the parasite's identification in stained swabs of blood and lymph nodes. (Nayel et al., 2012) The life cycle of the parasite is complex as it reproduces sexually in the intermediate host (ticks) and asexually reproduces in the vertebral host (ruminants) (Gohaaiz et al., 2016). Seven species of the thallaria parasite, Theileria annulata and T. parva, have been identified as being among the most pathogenic types, and T. velifera is considered non-pathogenic (OIE, 2004), T. taurotragi and T. mutans, where these two cause mild disease, T. sergenti and T. buffeli. These species differ with each other in pathogenicity, morphology, biological and molecular aspects, but the most important economically affecting cows and showing a high infection rate and mortality is Theleria. Parva that caused East Coast fever (ECF) And the second type, Theleria annulata, which causes Tropical Theileriosis. These two parasites have a continuous relationship with each other and from the newest parasites of the genus Theileria. Both T.parva and T.annulata cause similar pathological syndromes that are distinguished by lymphatic proliferation and an increase in the number of cases of the disease followed by deaths when Achieving an acute disease state similar to pulmonary infections and destruction of lymph tissue, but the main difference is that T. annulata affects the spinal cord and B lymphocytes, on the contrary, T. parva affects T lymphocytes (Allsopp and Allsopp 2006). Infection with the parasite Theileria

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annulata is one of the important and endemic diseases in Iraq, especially in the central and southern regions due to the spread of ticks, which are the vector host of the disease, where they are active and the infection increases in the summer and autumn seasons, as it increases when temperatures and humidity rise, Infection occurs in cows with a pathogen Tropical fever (theileriosis Tropical) or the Mediterranean coast fever (Mizaei, 2007).

This disease affects livestock as it causes non-growth of calves and thus delays their growth and causes a lack of milk production by cows in addition to poor meat, This disease affects in particular and significantly on imported cows to improve production because they are weak and sensitive to parasite infection. This parasite is one of the most important obstacles that affect livestock (Ahmed et al. 2008). The most important symptoms of the disease are enlarged lymph nodes, wasting, high temperatures (Hussein et al. 2004), sometimes diarrhea, weight loss, shortness of breath and pallor of mucous membranes (El-deeb Younis.2009).

Materials and methods

Area of the study

This study was conducted in Dhi Qar Governorate, which is located in the southern part of Iraq. This study included areas in Dhi Qar Governorate and its suburbs and villages that are next to the veterinary hospital, where samples were collected from September of 2020 to February of 2021, where samples were collected in randomize all ages for cows and for both sexes.

Collection Blood Samples

Blood samples were collected randomly, where blood was taken from the jugular vein of cows after sterilization of the area with ethanol alcohol (70%) (Chaudhri and Gupta, 2003), where (5 ml) of blood was drawn by a medical syringe (10 ml) and the blood is placed each in tubes containing an anti-blood clotting substance. (EDTA), where one of the part of blood (2.5 ml) is used for laboratory testing and making a slide for diagnosis and (2.5 ml) Keep it at a temperature of (-20 C) for the purpose of extracting DNA Laboratory diagnosis.

Laboratory of diagnosis:

To make a blood smear, in the first step, a clean glass slide was brought in and a drop of blood was placed on it after taking the blood by capillary tubes. The blood drop was placed on the edge of the slide, and the blood was brushed and pulled by another slide and placed over the drop of blood at an acute angle with the slide pulled quickly and lightly and then left The slide until the blood dried in the air for (1-3) minutes, ethanol alcohol (70%) was applied for 3 minutes, then the alcohol was disposed of and the Giemsa-stain dye was prepared by adding 9 ml of buffer solution to 1 ml to be (10%) of the dye (Chaudhri and Gupta, 2003) and then placed on the slide for 30 minutes to detect Theileria piroplasms, after which the slide was washed with water and allowed to dry and examined by light microscopy under magnification force X (100), where the annular structure of the peroplasm inside the red blood cell was observed

Molecular Study

DNA Extraction

DNA extraction was extracted from blood samples that were collected randomly, and by relying on the leaflet attached to the DNA extraction kit, DNA was extracted from the

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manufacturer Geneaid / USA, according to 1989 (Sambrook et al., Where it included the following steps

:**Material preoaration :**(100 ml) of Absolute ethanol was added to (25 ml) of the Wash Buffer solution, where the components of the mixture must be mixed together by shaking for several seconds and to keep the ethanol from evaporating, the bottle must be closed tightly. Add ddH2O (double distilled water) solution (1 ml) to the enzyme Proteinase K measuring (10 mg) to make the final concentration (11 mg / ml) and to ensure that it is completely dissolved, mix the components of the mixture with a Vortex device, then to ensure the rotation of the mixture that is at the bottom of the tube, we carry out the expulsion process. Centrifuge for a few seconds**.** Transfer (200 µl / sample) from Elution Buffer solution to (1.5 ml) from Eppendorf tube, where it is incubated in the water bath at a temperature of 60 ° C until reaching a step of adding it in DNA Elution

Preparation Sample Blood: Preparing the blood sample included the following steps: Transfer (200 µl) from tubes containing EDTA-K3 from frozen blood to Eppendorf tubes (pre-sterilized tubes) with a capacity of (1.5 ml). Add (20 µl) of Proteinase K enzyme and mix it well with a pipette . The tubes were incubated with a water bath for five minutes at 60 ° C . **Cell Lysis**: Add (200 ml) of the GSB solution after the end of the incubation period and mix the mixture vigorously by shaking. We return the tube Eppendorf to the water bath at 60 ° C for five minutes with continuous stirring of samples for two minutes.

DNA Binding: Add (200 µl) of Absolute ethanol to each sample with direct mixing using the Vortex device for 10 seconds, and if a thrombus appears, it must be broken directly using a pipette GS Columns were placed in Collection tubes of (2 ml) capacity . Transfer all the mixture, including any insoluble sediment, to the GS Columns. The centrifugation process was carried out to all samples at a speed of 14000-16000 rpm for a period of one minute, and in the event that the mixture did not exit through the GS Column membrane, the centrifugation time would be increased until it completely passed and the collection tubes containing the sediment were disposed of and replaced with new collection tubes. Collection with a capacity of 2 ml to which the GS Column tubes are transported.

Washing: Add (400 µl) of the W1 Buffe solution to the GS Column and centrifuge it at 14000-16000 rpm for 30 seconds, after which the sediment formed in the collection tubes was removed and transferred the GS Column to new collection tubes with a capacity of 2 ml. Then (600 micro-liters) of Wash Buffer (diluted with Absolute ethanol) was added to the GS columns. Conduct a Centrifuge process at a speed of 14000-16000 rpm for 5 minutes to completely dry the membranes of the GS columns to ensure the isolation of DNA and avoid any residual ethanol **Elution:** Transfer the dried GS columns to new Eppendorf tubes (1.5 ml). We add (100 micro liters) of preheated Elution Buffer in the center of the GS columns and then leave for at least 3 minutes to be absorbed completely. The Centrifuge process was carried out at 14000-16000 rpm for 30 s to ensure the isolation of the purified DNA.

Gel Electrophoresis : Deoxyribonucleic acid was detected by electrophoresis using Agarose gel at a concentration of 1% (Sambrook, et al. 1989). This method consists of the following materials:-(DNA, BufferTEB dewatering solution, Agarose 1%, Bromophenol blue, Ethedium bromide (10 mg / ml). It consists of the following steps:-**Prepartion of Agarose gel**: The concentration of agarose gel Agarose 1% was prepared, taking (1 g) of the acarose and

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dissolving it in (100 ml) (1x) of the buffer solution. Heat the solution to dissolve it using the microwave at a temperature of (55-60) degrees Celsius and make sure that the solution is completely dissolved. Leave to cool down at room temperature for 15 minutes. Then apply (1 µL) of Ethedium bromid dye.

Casting of Agarose gel: Pour the melted acarose into the tray of the acarose after closing its ends and after installing the comb comb at one end, which creates pits that are used to load the DNA samples and leave for 30 minutes until it freezes and solidifies at room temperature and when the solidification of the solution is complete, we lift the comb carefully and carefully Then we put the mold in the tank of the relay device after filling the tank with TBE (1x).

Loading and running DNA in Agarose gel :Putting (7 microliters) of the DNA sample and mixing it with (3 microliters) of Bromophenol blue until the volume became (10 microliters) and placed in the hole that is close to the negative electrode because the charge of the nucleic acid was negative. Then the electrodes of the device were connected and set to a voltage of (80 -100) for a period of one hour and notice the departure of the dye to the end of the acarose, and then transfer the template to the ultra-violet rays (UV) examination and view the DNA, where the result was photographed using a digital camera

Polymerase Chain Reaction (PCR) Technique

A PCR technique was used to detect Theileria annulata, for the cytob1 and 30-KDa genes. A pair of primers was used to amplify the cytob1 gene to 312 bp (Yang et al. 2015)

Forward: $^{5'}$-ACT TTG GCC GTA ATG TTA AAC–$^{3'}$
Reverse: $^{5'}$-CTC TGG ACC AAC TGT TTG G–$^{3'}$
A pair of primers was used to amplify the 30-KDa gene to 721 bp (d'Oliveira *et al*., 1995).

Forward: $^{5'}$-GTAACCTTTAAAAACGT-$^{3'}$
Reverse: $^{5'}$-GTTACGAACATGGGTTT-$^{3'}$

And through the leaflet attached to Green Master Mix by Geneaid, the mixture was collected to a volume (20 microliters) without calculating the AccuPower® PCR PreMix pellet tubes as shown in Table (

PCR Master mix	**Volume**
DNA template	5µl
Forward primer (10pmol)	1 µl
Reveres primer (10pmol)	1 µl
PCR water	13 µl
Total volume	20µl

After all the additions were made, the samples were placed in a vortex centrifuge shaker to mix all the components together, then these samples were placed in the Thermal Cycle device and the program ran the following steps:

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Table (2) Conditions for rotating the PCR device

No. of Cycle	Time	Temperature	Steps
1	5min	95C	Initial Denaturation
30	30sec	95C	Denaturation
	30sec	58C	Annealing
	1 min	72C	Extension
1	5min	72C	Final extension
	4C	Forever	Hold

PCR Product Analysis

Upon completion of the PCR program, the products are migrated by preparing (2%) of acarose by placing (1.2g) of acarose in 60 mL of TEB buffer solution with the same electrophoresis steps of the base pair 312 bp (Yang et al. 2015; Bampali et al., 2015) and this indicates the presence of the gene cytob1 through the use of Bioneer Daejeon, 1 (Kb ladder) South Korea).

Results and discussion:

The results of the current study showed that after the diagnosis of theileria parasite in the blood of cows, the infection rate was 19%, depending on the direct blood smear method, while the infection rate was 50% using the pcr technique, using the cytob1 gene of 312 pb and the 30-KDa gene of 712pb. When comparing the two methods with diagnosis, it was found that the PCR technique is more accurate than the direct blood smear method. Fig (1, 2, 3 and 4)Table (3)

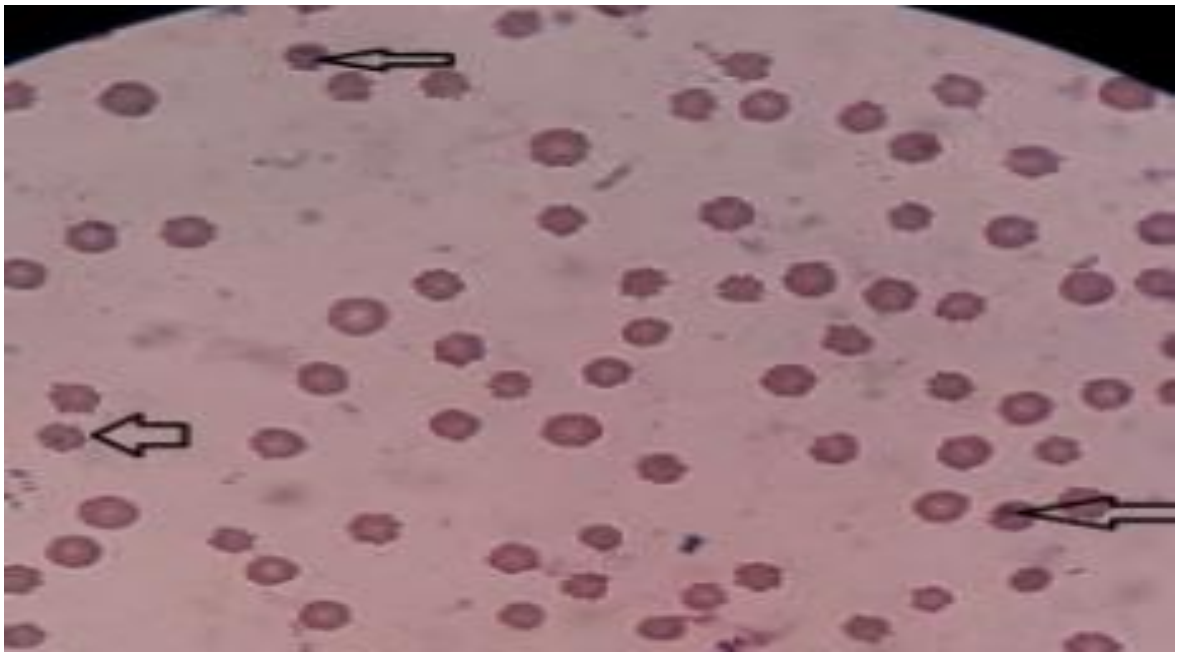

Figure (1) illustrates the T. annulata in the direct smear of the blood sample of infected cows (100x)

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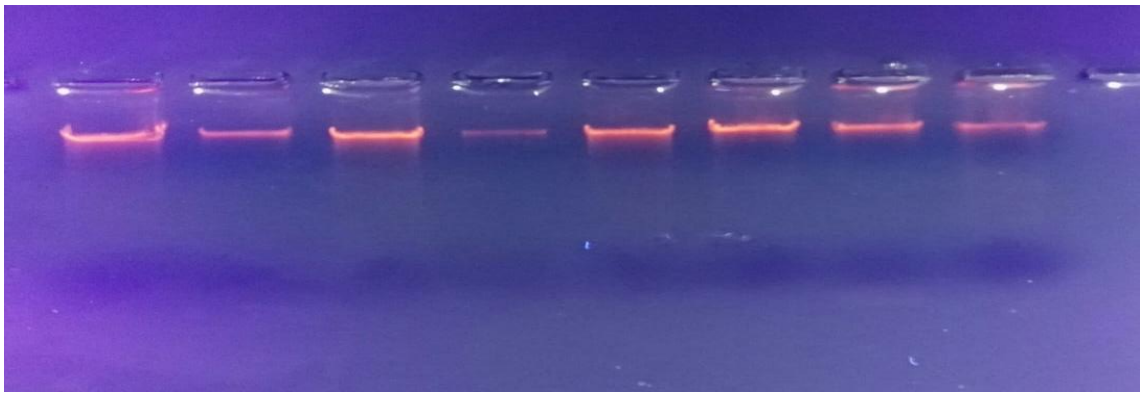

Figure 2: Agarose Gel Electrophoresis Image shows amplified DNA extraction of T.annulata in blood samples from cow

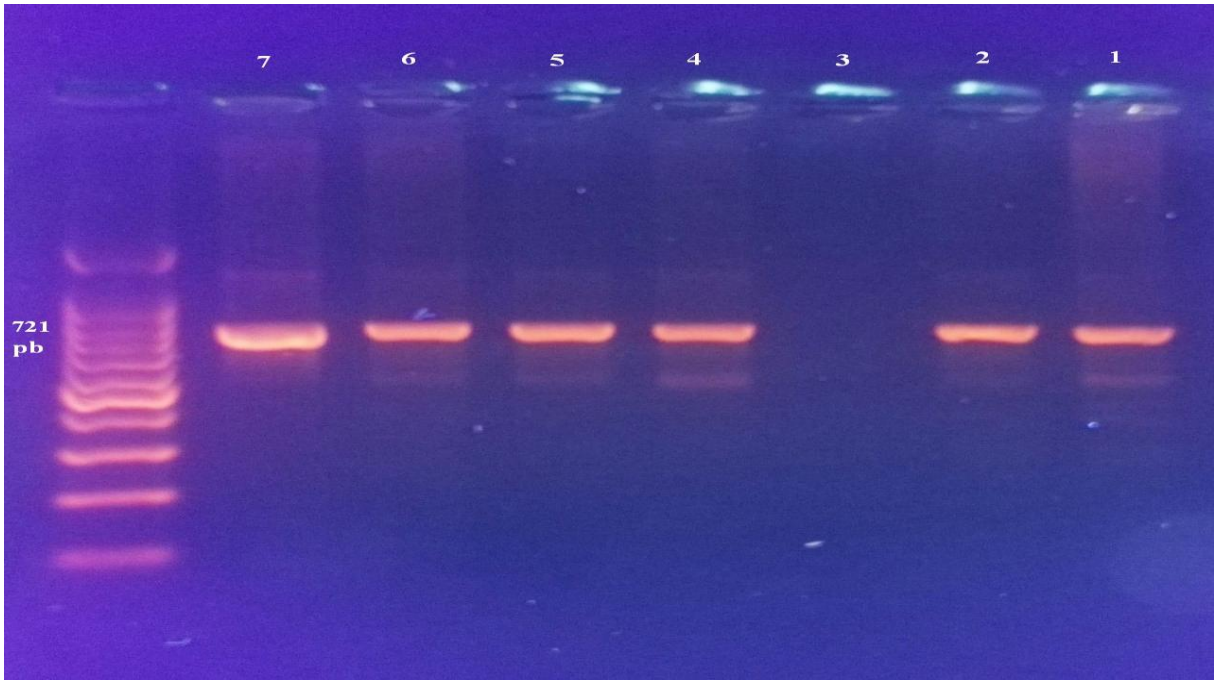

Figure 3: Detection of *T. annulata* for gene with size 721 pb in blood samples by the PCR assay. The figure shows results of agarose gel electrophoresis of amplified DNA extracted from blood samples obtained in the present study. Lanes 1, 2, 4,5,6,7 infected blood samples; lanes 3 non-infected blood samples; lane 8 molecular size marker.

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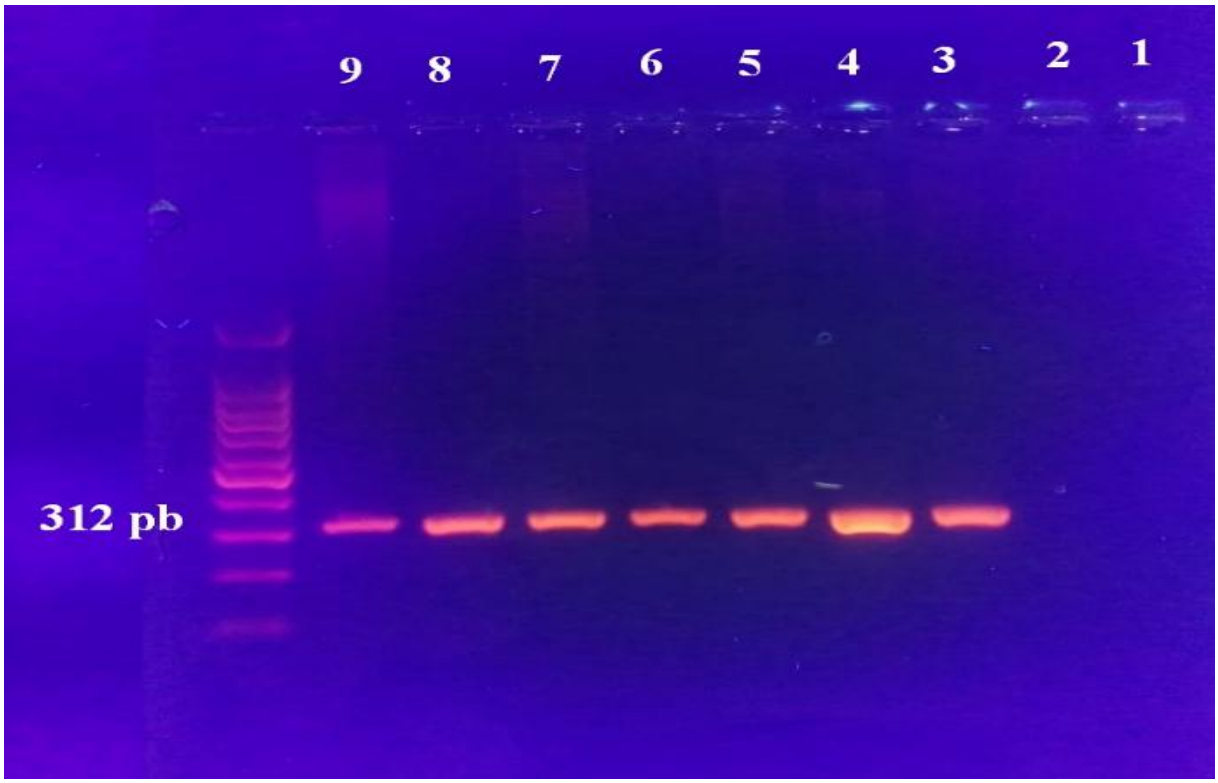

Figure 4: Detection of *T. annulata* for cytob1 gene with size 312pb in blood samples by the PCR assay. The figure shows results of agarose gel electrophoresis of amplified DNA extracted from blood samples obtained in the present study. Lanes 3-9 infected blood samples; lanes 1-2 non-infected blood samples; lane 10 molecular size marker.

Table (3) Comparison of the two methods for diagnosing parasites by PCR and direct smear method

Diagnostic method	Examined	Infected	%
Smear method	214	41	19%
PCR	100	50	50%

X^2 cal. 31.50 df. 1 $P < 0.05$

The current study showed significant different between smear direct and PCR to diagnose bovine theileria, the incidence of bovine theileria was relatively high 19%, as (41/214) was diagnosed with a light microscope with a magnification power (100x) using the Geimsa stain, where the annular structure of the peroplasm was seen inside the red blood cell, which is in the form of an annular or a comma. Inside the red blood cells (Mandal et al. 2006)), as well as the apparent change in the shape of the globule due to the presence of the parasite inside it, and the results of the microscopic examination of this study are close to that of the study

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carried out by (Nayel et al. 2012), where this study was conducted in Egypt, which It was found (19%) was positive, and this result was close to the results obtained by (Tallaf et al. 2017) that were conducted in Muthanna Governorate, southern Iraq, where (27/204) blood samples were collected and a microscopic diagnosis was (13.23%). And it was close to what was presented by Al-Abadi and Al-Badrani (2012), where the study was conducted in the province of Mosul, northern Iraq, and the infection rate was (13.63%) from (220/30) and was the result of a study that occurred in Iran (Nourollahi-Fard et al., 2013) where the percentage of laboratory diagnosis of bovine theileria was (10.66%) as it was diagnosed (16/150). While the result of a study that took place in Algeria was Ziam et al. (2015) (11.69%) people through this study (42/359) and that the reason for the decrease in the incidence rate as a result of laboratory examination was also stated by Ziam et al. (2015) that the reason for the decrease in infection is due to a decrease In the rate of infection with the average host of ticks for the vector, as it is affected by humidity and temperature, especially since the months of the study extended from September of 2020 to April of 2021, and on the other hand, these results were much less than the results that emerged from (Ali and Nabil et al. 2012). It was conducted in northern Iraq in Sulaymaniyah governorate, where (50) blood samples from cows were examined, and a laboratory examination was conducted, where the annular structure was seen inside the red blood cells of 27 samples (54%) and it was not consistent with the study conducted in Baghdad governorate Hameed et al. 2019)) from before, where 150 samples were collected, through microscopy, 59 (59/150) positive blood smears were diagnosed for the presence of Theleria annulata parasite by (39.33) and it was less than the result obtained by each of Lkhaledi et al. 2008) at (32.93), as well as Al-Emarah et al. 2012) (69.43%), where they explained that the reason for the discrepancy in the rates of laboratory diagnosis is that the intermediate host of ticks needs warm temperatures and warm weather for reproduction and explain Muhammad et al. (2008) Cattle differ in their resistance to ticks, and thus their degree of susceptibility to disease differs due to the innate immunity to different infections from one cow to another.

Our study showed the percentage of direct smear diagnosis of 19% compared to the pcr method 50% and compared to a study conducted in Baghdad by Al-Samarai el at.2019)) 96 blood samples were examined by a microscope and with a magnifying glass 100 X with slices dyed with Camza dye, where the result of the examination was The diagnosis of annular peroplasm in red blood cells 96/32 with a ratio of (33.33), whereas in healthy cattle no annular structure was observed, while the result of the pcr test was 57.29% (55/96), as this

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result was higher than that obtained by Alkhaledi, (2008) in Iraq, which was the prevalence (32.93%), where Al-Abadi and Al-Badrani (2012) scored in Mosul (13.63%) it was (120/30), while the result in neighboring countries was higher than in Iraq, where it was in Saudi Arabia. The prevalence of bovine fever (15.38%), while in Iran (10.66%) and Algeria (11.69%) (Omer et al, 2003; Nourollahi-Fard et al, 2013 and Ziam et al, 2015), on the other hand, the average of the study conducted by Al- Samarai el at.2019.((

A study in Baghdad governorate by (Hameed et al 2019) showed that 25 cows blood samples were examined using the polymerase chain reaction technique, where the parasite was detected in 22 infected samples, where the gene (cytochrom b) was amplified (312 bp) in Baghdad governorate, and the result was By (80%), while the study conducted in the Kurdistan region in 2009 by (Adel et al. 2009) showed a significant increase in the incidence of bovine telluria fever in the three governorates, where the infection rate reached (62.6%) for Erbil governorate and (69%) for Duhok governorate and (74%) for Sulaymaniyah governorate and in a study conducted in Muthanna governorate (Kamel et al. 2017) showed a high rate of infection 33% was positive for ring theileria. These results were less than those recorded in the Kurdistan region, where they were The infection rate was 68.9% (Al-Saeed et al 2010), in addition to the results (Al-Emarah et al. 2012) in Basra Governorate, where the rate was 88.23%.

In a study in Egypt (Muhammad et al. 2018) that (11.44%) of cattle were positive for infection, as these results agreed with Ibrahim et al. (2009), as the percentage of cattle infected with tropical fever was (13%), while Elsify et al found (2015) that (9.56%) were examined in different regions of Egypt, while Al-Hosary et al. (2015) found the incidence of infection with a diagnostic medium with the pcr technique (73.29) that the polymerase chain reaction technique revealed accurately (7.5%) in Golestan. In Iran, Hoghooghi-Rad et al. (2011) was positive and in a study in India, Chauhan et al. (2015) recorded (46.15%) of the cows carrying the disease.

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