Archives of Razi Institute, Vol. 78, No. 1 (2023) 389-396

DOI: 10.22092/ARI.2022.359033.2358

Original Article

Quantification of Acute and Chronic Theileriosis in Sheep by Quantitative PCR (qPCR)

Rassim Mohammed, A[1 *], Al-Saadi, M[1]

1. Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Qadisiyah, Iraq

Received 13 June 2022; Accepted 10 July 2022
Corresponding Author: naeem.2t@yahoo.com

Abstract

Small ruminants theileriosis are widespread in Iraq andacute infections usually with hight mortality. However, the survived animals suffer from low production of meat and milk. Coinfection with more than *Theileria sp.* And/or Anaplasmosis could have an impact on the disease severity. The main finding was identifying *T. lestoquardi, T. ovis , T. annulata,* blood samples of infected sheep with a history of chronic theileriosis (n=48) and with acute clinical theileriosis sign (n=24) were being collected from fields located in Babylon province (middle of Iraq) after chlinical examination and Polymerase chain reaction and real time PCR were performed for detection. *Theileria. lestoquardi* was the highest of these species within the acute and chronic cases. As well as, the load of this species in acute cases was significantly higher (P<0.01) to that in chronic. However, the load of *T. ovis and T. annualta* were similar in acute and chronic cases. Importantly, all these cases were coinfected with *Anaplasma phagocytophylum.* This could be due to the infection of leukocytes meanwhile weakening of the animal's immune system. Also, these parasites transmitted by the same tick-vector. The impact of this finding could help in disease prevention and diagnosis.

Keywords: *Theileria sp. , Anaplasma phagocytophylum,* Real time PCR, Iraq

1. Introduction

Among many tick-borne pathogens, theileriosis (as a Piroplasmida) and anaplasmosis (intracellular Gram-negative tick-transmitted bacterialpathogen) are both endemic and cause serious problems in Iraq (1).Tick-borne diseases (TBDs) are extremely important in the veterinary medicine (2). In tropical and subtropical zones, including Iraq, tick-borne hemoporotozoan and rickettsial pathogensinhibit animal productivity (3). Tick-borne diseases such as anaplasmusis and thieleriosis are serious diseases caused by bacteria and blood parasites transmited by ticks (4). This threatens the small ruminant breeding and livestock economy as a result of decreased productivity and high mortality rate (2). *T. lesteoquardi, T. luwenshuni* and *T. uiolenbergi* which cause high mortalities, cause theileriosis in small ruminants, but *T. ovis* and *T. annulata* are less harmful (5). In the Arabian Peninsula, *Theilereia lestoqouardi* is one of the most common and pathogenic Theileria spp. of small ruminants, causing significant illness and death among indigenous sheep in Iraq (6). *Theileria lestoquardi* is the cause of the malignant form of theileriosis, in which infected Animals either do not respond to treatment or have poor conditions and productivity (7). The severity of these infections is depended by the parasite virulence, infectious dose, animal breed, and animal's immunological condition (8, 9). Furthermore, Anaplasmosis is a tick-borne disease that causes health problems and reduces the value of livestock (10). Tick-

borne pathogens *Theileria spp*. and *Anaplasma phagocytophilum* all target the host's leukocytes (both reduce host immunity) (11). In general, when animals recoverdfrom acute or primary infections after treatment, some parasites often survive, a condition known as carrier state, which is generally undetected under the microscope but allows transmission by the tick vector (12). The carrier state increases the risk of disease spread into places where the vector is present, even if the disease is not present.

Theileria spp. chronic asymptomatic is associated with severe losses in animal productivity (13). In this study, it was hypothesized that presence of malignant and/or chronic thieleriosis may appear due to either coinfections with more than one thielerial species and/or with anaplasmosis. The main aims of the study are demonstrating the coinfection of theileriosis with *Anaplasma phagocytophilum* as all are tick-borne pathogens that targeting host leukocytes (both reduce host immunity). Measuring the parasitism load and detect the coinfection with bacterial infection that targeting the leukocytes within acute and chronic cases of sheep helps to identify the main causative agent of the disease.

2. Materials and Methods

2.1. Sample Collection

Whole blood samples of infected sheep with a history of chronic theileriosis (n=48) and with acute clinical theileriosis sign (n=24) were being collected from fields located in Babylon province (middle of Iraq) during the periodfrom October 2021 to January 2022 for chronic cases and from April-May 2022 for acute cases. Blood samples were taken from sheep's jugular veins in tubes containing EDTA anticoagulant, then transferred to the laboratory in a cold box for DNA extraction.

2.2. The DNA Extraction

Genomic DNA was extactedfrom the blood samples by using of Geneaid (Korea) Kit, as directed by the manufacturer. Briefly, the RBCs were destructed with lysis buffer then the WBCs were lysed meanwhile the lysates containing DNA were purified with spin column and washing buffers. Finally, the spin column was used to elute the DNA by 50 ul of elution buffer. These were kept at -20C until further analysis.

2.3. Molecular Detection Methods

2.3.1. Polymerase Chain Reaction (PCR) Amplification

PCR primer sequences and cycle procedures for the identification of *T. lestoquardi*, *T. ovis*, and *T. annulata* based on Habibi, Sepahvand-Mohammadi (14). while the procedures to detect *A. phagocytophylum* were utilised from Yang, Liu (15).

Three primers were used to amplify a particular region within *Theileria* sp. (14). These primers were selected as qPCR primers to modify conventional PCR to qPCR after assay validation as shown in the table 1.

Detection of *Anaplasma phagocytophilum* by nested PCR was done (Table 2).

Table 1. Primer for identification of each Theileria spp. in qPCR assays

Theileria spp	Forward	Revers	Size (bp)
Theileria annulata	GAGACAAGGAATATTCTGAGTCC	TTAAGTGGCATATAATGACTTAAGC	547 bp
Theileria ovis	GTAGGGCTAATACATGTTCGAGACCTTC	TGATACATCGCATCCGAAGAC	121 bp
Theileria lestoquardi	ATCAGCGGCAACACAACC	TTCCTGGTCATGAGAACCG	400 bp

Table 2. Primer used in the nested PCR experiments for detecting *A. phagocytophylum*

Primer name	First round		Size (bp)
	Forward	Reverse	
A. phagocytophylum	TCCTGGCTCAGAACGAACGCTGGCGGC	AGTCACTGACCCAACCTTAAATGGCTG	1200 bp
	Second round		
	GTCGAACGGATTATTCTTTATAGCTTGC	CCCTTCCGTTAAGAAGGATCTAATCTCC	926 bp

This was carried out by conventional gradient PCR to detected the optimal temperature of each *theileria spp* ,in which used a gradient procedure with a final reaction volume of 20 µl that contained 11.8 µl water , 2 µl of 10x buffer, 2 µl of dNTP,1 µl of each primer, 0.2 µl of Taq polymerase, and 2 µl DNA . In an automated thermal cycler, the PCR profile consisted of a 10-minute denaturing stage at 95°C, then 38 cycles of denaturing at 95°C for 35 seconds, gradient annealing at 55-65°C for 30 seconds, and extension at 72°C for 30 seconds. Amplicons were visualised by agarose gel electrophoresis (1.5 %) in which the DNA was stained by ethidium bromide.

2.3.2. Real Time (q PCR) for Amplification *theileria spp*

The test was performed in a total reaction volume of 20 µl that contained 10µl of sybergreen master mix (AddBio, South Korea), 1µl of each primer (Table 1) ,2 µL of template DNA, and 6 µl of PCR water. The amplification conditions were carried out using qPCR machine (Applied Biosystem, USA) as a one cycle of 95 °C for 10 min followed by 38 cycles of 95 °C for 15 seconds as denaturation, 55 °C for 35 seconds of annealing, and 72 °C for 30 seconds of annealing, followed by a five-minute extension at 60 °C.

2.3.3. Conventional PCR Reaction Mix and Thermal Cycling for Amplification of *Anaplasma phagocytophilum*

The presence of *A. phagocytophilum* was determined using nested PCR on the isolated DNA. The reaction of nested PCR in first round, was carried out in a Bio-Rad automated theremocycler with a total capacity of 20 µL containingthat contained 10µl of Master mix, 1µl of each primer (Table 2), 2 µL of template DNA, 6 µl of PCR water. The samples were combined, spun lightly, and placed in a thermocycler. For 38 rounds, the reaction was repeated.

Nested PCR insecond round was performed as in the first round except amount of DNA (1 µL from first round) and inner primers (Table 3). PCR thermal conditions were done same in the first and second round by using conventional PCR thermocycler system. DNA amplification was carried out in Prime Thermal cycler, the program was 95 °C/5 min, followed by 35 cycles, 95 °C/35 seconds for denaturation, 55 /30sec for annealing, and 72 °C/60 sec, with a final extension at 72 °C for five minutes. Amplicons were observed on an agarose gel (1.5 %) in which the DNA was stained by ethidium bromide.

Table 3. Stat summary for chronic cases of theileriosis

Theileria sp	*T. annualta*	*T. ovis*	*T. lestoqouardi*
Number of values	48	48	48
Minimum	0	20.52	15.13
Maximum	26.98	28.23	24.45
Range	26.98	7.71	9.32
Mean	25.06	24.8	18.57
Std. Deviation	3.872	1.694	2.595
Std. Error of Mean	0.5588	0.2445	0.3746

2.4. Statistical Analysis

The data of this study were analyzed according to the program of analysis variance by using SPSS software statistical program (SPSS for windows ver.17.00). Quantification of theilerial coinfection by qPCR analysis within the acute and chronic infection was statistically analysed by one-way ANOVA and un-paired t test. The difference in mean values was considered as significant at ($P \leq 0.05$).

3. Results

3.1. Clinical Examination Results

As a result of chlinical examination in chronic cases, it was found that in most sheep, the mucous membranes of the eyes and mouth are pale and also the lymph nodes, especially the lymph nodes of the femur and forearm have enlarged. In the most animals were emaciatedtheir hair were curly, the temperature ,pulse rates and respiration rate mostly normal.clinical examination in chronic cases included, temperature 39-40.6 ºC, pulse rate 72-86, and respiration rates 22-34, in addition to pallor of the mucous membranes of the eyes and mouth, and enlarged lymph nodes.

However, in severe cases, clinical examinations were clear and distinct by noting high temperature range

40.5-41.6 ºC, pulse rate 82-85, resoration rate 32-35, mucus membrane state were paleness and Lymph nodes palpation enlargement.

3.2. qPCR Optimization Results

Initially gradient protocol was carried out by conventional gradient PCR in which all the temperature ranges were optimal. thus annealing temperature of each *theileria* spp (*T. lestoqoardi, T. ovis, T. annulata*) was 55º C as in figures 1, 2 and 3.

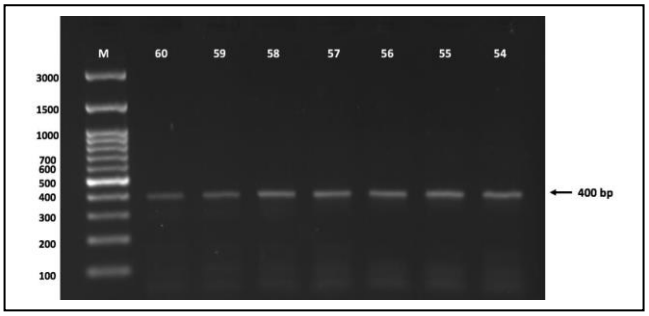

Figure 1. Gel electrophoresis image (1.5 % agarose) shows the amplicons of *Thieleria lestoqouardi* (size=400 bp) by gradient protocol. M is molecular marker from Genedirex, Korea

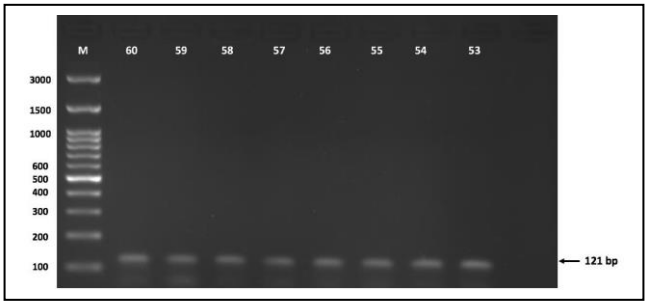

Figure 2. Gel electrophoresis image (1.5 % agarose) shows the amplicons of *Thieleria ovis* (size= 121 bp) by gradient protocol. M is molecular marker from Genedirex, Korea

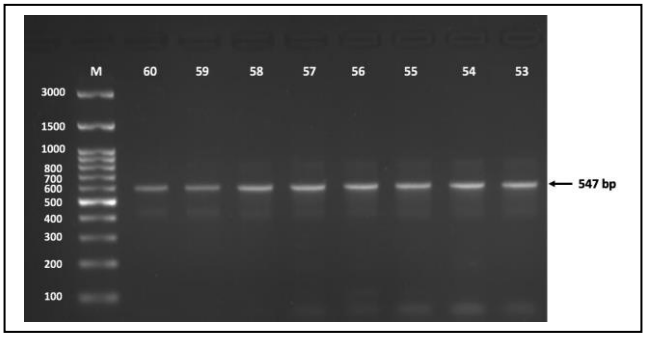

Figure 3. Gel electrophorsis image (1.5 % agarose) shows the amplicons of *Thieleria annulata* (size= 547 bp) by gradient protocol. M is molecular marker from Genedirex, Korea

3.3. qPCR Quantification Results

3.3.1. Quantification of theileria spp (*T. lestoquardi, T. ovis, T. annulata*)

48 samples of chronic cases of sheep was tested for the theileriosis by qPCR thermocycler system, all of these cases was infected with *T. lestoquardi, T. ovis,* and *T. annulata*. The CT values of *T.lestoquardi* was lowest than to that of *T. ovis*, and *T. annulata* that indicative high parasitism load and high virulent .as the highest CT value of *T. lestoquardi* was (24.45), the lowest was (15.13). While the highest CT value of *T.ovis* and *T.annulata* was (28.23, 26.98) and the lowest was (20.52,0) respectively, as in figure 4. In qPCR run assay specify was also checked by melting analysis as in figure 5.

3.3.2. Quantification of *theileria* sp Coinfection in the Acute and Chronic Cases

The three *Theileria spp*. were found in all of the samples (*T. lestoquardi, T. ovis, T. annulata*) except one sample was not infected. Importantly quantification of *T. lestoquardi* was the highest infection as the CT value was lower than to that of *T. ovis* and *T. annualta.* indicative of the high parasitic loads of this species in both chronic and acute cases, as in tables 3 and 4.

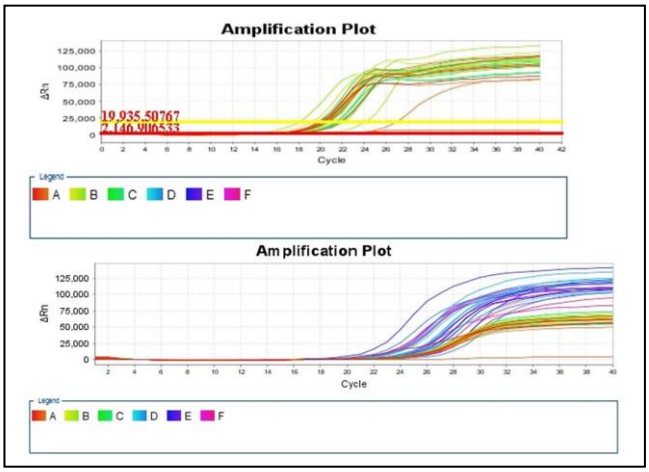

Figure 4. qPCR images shows the amplification curves of *T. lestoquardi* ,*Theileria ovis* and *T. annulate* of infected samples

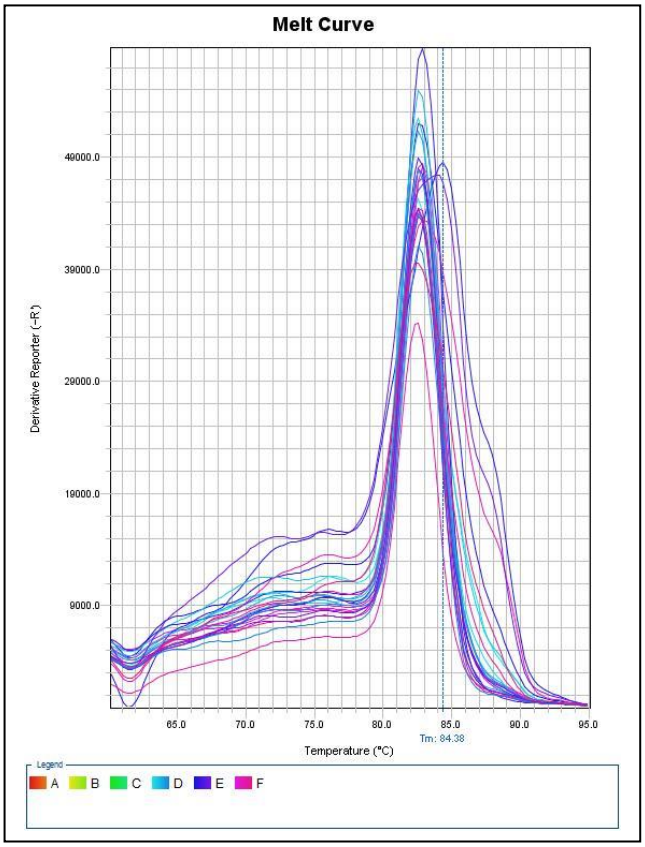

Figure 5. Melting curve analysis of the amplified products from qPCR shows one peak of all positive amplicons indicating successful qPCR optimisation without non-specific amplification

Table 4. Stat summary for acute cases of theileriosis

	T. annualta	*T. ovis*	*T. lestoqouardi*
Number of values	24	24	24
Minimum	16.27	20.02	11.3
Maximum	31.81	29.93	16.1
Range	15.54	9.91	4.8
Mean	23.54	24.64	14.23
Std. Deviation	4.136	2.279	1.3
Std. Error of Mean	0.8442	0.4652	0.2654

In the chronic and acute cases, there was a significant relationship between *T. lestoquardi* and *T. ovis* and between *T. lestoquardi* and *T. annulata*. While there was no significant relationship between *T. ovis* and *T. annulata* as in figure 6. On the other hand, there was a significant relationship between *T.lestoquardi* in chronic and acute cases, while the relationship between the *T .ovis* in chronic and acute cases was non-existent, as with *T.annulata* in both chronic and acute cases. Importantly, in chronic cases the cT values of *T. lestiquirdi* were significantly lower than to that of *T. ovis* and *T.annualta*. This indicating a higher parasitisim load compared with the two species. Importantly, in acute cases the CT values of *T. lestiquirdi* were significantly lower than to that of *T.ovis* and *T.annualta*.The cT values of *T. lestoqouardi* in acute and chronic infections were significant butin *T. ovis and T. annulata* in acute and chronic infections were non-significant (P<0.05) (Figure 7).

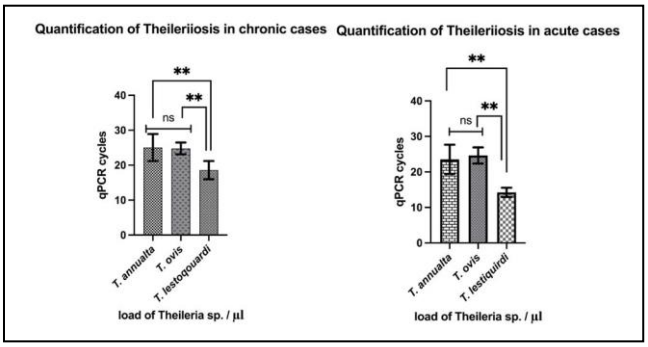

Figure 6. Quantification of theilerial coinfection by qPCR analysis within the chronic and acute infection

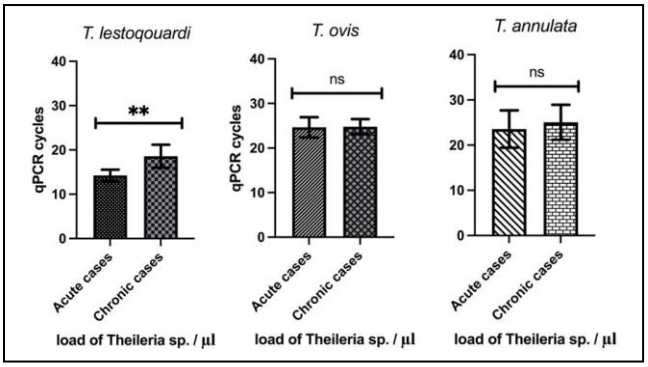

Figure 7. Quantification of *T. lestoqouardi, T. ovis and T. annulata* by qPCR analysis within the acute and chronic infections

3.4. Coinfection of *theileria* sp with *Anaplasma phagocytophylum*

Importantly, all the quantified *theileria* sp were found positive for the infection of *Anaplasma phagocytophilum.*All were positive inthe acute and chronic infections, as in figure 8.

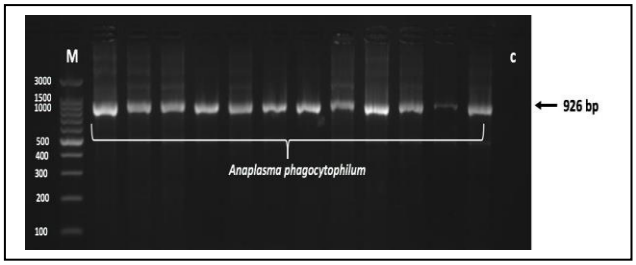

Figure 8. Gel electrophoresis image (1.5 % agarose) shows the amplicons of *Anaplasma phagocytophilum (*size= 926 bp). C is negative control in which similar PCR conditions were used except H_2O was added instead of DNA. *M is* molecular marker from genedirex

4. Discussion

Ovine theileriosis is one of the most serious diseases affecting small ruminants, especially in the tropics and subtropics, including Iraq, where it causes economic losses in sheep and goats (16). Currently, Theileriaspecies and *Anaplasma phagocytophylum* are endemic in sheep in Iraq. These cases were mostly suffering from severe infections of theileria, especially in young ages. These mostly characterised by a high mortality rate and poor response to treatment. Importantly, the recovered cases continued to suffer from delayed growth, weight loss, wasting and scalping fleece as it turned into the chronic form of the disease. Sheep become chronic carriers of tick-borne disease and serve as reservoirs for transmission of infection As a result, latent infections are crucial in disease epidemiology (17). In this study, Chronic and acute cases of theileriosis were being collected to find out the factors leading to the severity of the infection, the presence of pathogens and its species. Traditional diagnosis of piroplasmosis is less valuable to detect chronic cases. However, PCR technique has advanced the approach in the detection even with low parasitism. Here in, we developed qPCR quantification assay using primer designed for conventional PCR by Habibi, Sepahvand-Mohammadi (14). In this study the parasitism loads were high for T.lestoquardi indication of its virulence, where the highest CT value was (24.45, 16.1) and the lowest CT value was (15.13, 11.3) for chronic and acute cases, respectively. While the parasitism loads for T. ovis and T. annulata were similar, where the highest value was (28.23,29.93,26.98,31.81) and the lowest value was (20.52,20.02,0,16.27) in the chronic and acute cases, respectively. The obtained results revealed that coinfections including, three thieleria species (*T.lestoqouardi ,T.ovis and T. annalata*) and one species of anapalasma (*Anaplasma phacocytophylum*) were detected in sheep. Importantly, *Theileria lestoquardi* was found the highest infection load. Malignant ovine theileriosis is aserious lymphoprolifestive disease that causes a high rate of morbidity and mortality in sheep, is caused by this theileria species, which is the most virulent in goats and sheep (18). In Oman, Al-Hamidhi, Elshafie (19) found that coinfection with the pathogenic Thieleria lestoqoardi and the non-pathogenic Thieleria ovis was detected in a high percentage of sheep, exhibiting an interaction and decreased mortality risk in mice infected with *T. lestoqardi* and *T. ovis.* This implys that the latter protects against *T. lestoquardi* pathogenicity.

In a research by Heydarpour, Khazraeinia (20), found that 55.3% positive for *T. lestoquardi*, while 44.7%samples tested positive for *T. ovis.* The quantification data regarding the coinfection was found similar to that previously obtained by Hegab, Fahmy (21) that demonstrated at least three genetically distinct *Thieleria* spp. found in Egyptian sheep. (*T. ovis, T. lestoqoardi and T. annulata*). Additionally, *Anaplasma phagocytophylum* was reported to be present in all *theileria sp.* positive samplesimplying a complex Pathogenicity. Ceylan, Byamukama (22) found that at least one of the protozoan and rickettsial illnesses was confirmed in *B. ovis, A. ovis, T. ovis,* and *A.phagocytophilum.* In a study conducted by Renneker, Abdo (23). In Iraq in the Kurdistan region, it showed that 23% (45/195) of the tested samples contain more than one species of pathogens (*anaplasma.ovis, T.ovis, T. lestoqardi, T. uilenebergi,* and *B. ovis*).

The phenomena of numerous infections in animals is a topic that has received little attention to date. Khayyat and Gilder (24) described coinfection of small ruminants in Iraq for the first time in 1947, but no

further research has been done to our knowledge. Coinfection of sheep with various diseases was discovered in this investigation. Overall, all sheep samples were infected with more than one virus, which exacerbates the animals' health problems, As a result, as observed by Khayyat and Gilder (24) the loss of products occurs, indicating that superinfection by other hemoparasitic species occurs and that there is no cross protection between these species.The current findings revealed the presence of *T. annulata* in sheep in Iraq implying that this pathogen has a broader range of hosts than previously thought. *T. annulata* has only been found in cattle so far, and it is considered very pathogenic (25). *Theileria annualta* had less load than *T.lestoqardi* and *T.ovis*, according to the quantification data in both chronic and acute cases.

The molecular study conducted in Iran by Zaeemi, Haddadzadeh (16) showed for the first time the presence of *T. anulata* in the samples of sheep infected with *Theileria sp.*, where these samples were examined by nested PCR-restericton fragment length polymorphism (RFLP) technique, and the results were the presence of *T. annulata* as a co infection with *T. lestoquardi and T.ovis*. Sequence analysis confirmed the presence of *T. annulata* .The presence of high load of *T. lestoquardi* in chronic and acute cases demonstrate its role in ovine theileriosis. Moreover, the load in acute cases was significantly higher than to chronic infection. Indicating that the season of tick-vectors play an important role to increase the parasitism of *T. lestoquardi* and leading to theileriosis.

This study concludes that qPCR is a power tool to quantify these parasites. Also, can be used to detect coinfection in a less time and efforts. all the quantified samples revealed the infection with *Anaplasma phagocytophylum.* Howver, its role in the severity and chronic cases are still unclear. The environmental conditions likely have a role in increasing the production of more ticks that serve as a vector for theileriosis and anaplasmosis and possibly these two-parasite transmitted by the same tick species or more than one. The severity of the infection is caused by infection with more than one species of theileria (*T. lestoquardi*, *T. ovis*, *T. annulata*) and *Anaplasm phagocytophylum*, which all infect white blood cells and thus lead to weakening the immune system, causing high mortality rate.

Authors' Contribution

Study concept and design: A. R. M.
Acquisition of data: M. A. and A. R. M.
Analysis and interpretation of data: A. R. M.
Drafting of the manuscript: A. R. M.
Critical revision of the manuscript for important intellectual content: A. R. M.
Statistical analysis: M. A.
Administrative, technical, and material support: M. A.

Ethics

The study protocol was approved by the ethics committee of the University of Al-Qadisiyah, Al-Qadisiyah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Stuen S. Haemoparasites in small ruminants in European countries: Challenges and clinical relevance. Small Rumin Res. 2016;142:22-7.
2. Uilenberg G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. Vet Parasitol. 1995;57(1-3):19-41.
3. Sevinc F, Xuenan X. Major tick-borne parasitic diseases of animals: A frame of references in Turkey. Eurasian J Vet Sci. 2015;31(3):132-42.
4. Zhou M, Cao S, Sevinc F, Sevinc M, Ceylan O, Moumouni PFA, et al. Molecular detection and genetic identification of Babesia bigemina, Theileria annulata, Theileria orientalis and Anaplasma marginale in Turkey. Ticks Tick Borne Dis. 2016;7(1):126-34.
5. Goudarzi G, Tavakoli M, Ezatpour B, Kooshki H, Hosseini-Chegeni A. Molecular detection of Theileria ovis (Apicomplexa: Theileriidae), Anaplasma ovis

(Rickettsiales: Anaplasmataceae), and Mycoplasma sp.(Tenericutes: Mycoplasmataceae) from sheep blood in western Iran. Comp Clin Path. 2019;28(6):1661-6.
6. Tageldin M, Fadiya A, Sabra A, Ismaily SA-I. Theileriosis in sheep and goats in the Sultanate of Oman. Tropical Animal Health and Production. 2005;37(6):491-3.
7. Alkhaled M, A'aiz N, Naser H. Phylogenetic study of Theileria lestoquardi based on 18SrRNA gene Isolated from sheep in the middle region of Iraq. Iraqi J Vet Sci. 2016;30(2):27-32.
8. Ayadi O, Gharbi M, Benchikh-Elfegoun MC. Haematological and biochemical indicators of tropical theileriosis diseased cattle in wilaya of Sétif (North East Algeria). J Parasit Dis. 2017;41(2):538-42.
9. Singh SK, Roeffen W, Mistarz UH, Chourasia BK, Yang F, Rand KD, et al. Construct design, production, and characterization of Plasmodium falciparum 48/45 R0. 6C subunit protein produced in Lactococcus lactis as candidate vaccine. Microb Cell Fact. 2017;16(1):1-11.
10. Aouadi A, Leulmi H, Boucheikhchoukh M, Benakhla A, Raoult D, Parola P. Molecular evidence of tick-borne hemoprotozoan-parasites (Theileria ovis and Babesia ovis) and bacteria in ticks and blood from small ruminants in Northern Algeria. Comp Immunol Microbiol Infect Dis. 2017;50:34-9.
11. Jenkins C. Bovine theileriosis in Australia: a decade of disease. Microbiol Aust. 2018;39(4):215-9.
12. Al-Fahdi A, Alqamashoui B, Al-Hamidhi S, Kose O, Tageldin MH, Bobade P, et al. Molecular surveillance of Theileria parasites of livestock in Oman. Ticks Tick Borne Dis. 2017;8(5):741-8.
13. Gharbi M, Touay A, Khayeche M, Laarif J, Jedidi M, Sassi L, et al. Ranking control options for tropical theileriosis in at-risk dairy cattle in Tunisia, using benefit-cost analysis. Rev Sci Tech. 2011;30(3):763.
14. Habibi G, Sepahvand-Mohammadi E, Afshari A, Bozorgi S. Molecular detection of Theileria spp. and Babesia ovis Infection in Sheep in Baneh, Iran. Arch Razi Inst. 2020;75(2):289.
15. Yang J, Liu Z, Niu Q, Liu J, Xie J, Chen Q, et al. Evaluation of different nested PCRs for detection of Anaplasma phagocytophilum in ruminants and ticks. BMC Vet Res. 2016;12(1):1-6.
16. Zaeemi M, Haddadzadeh H, Khazraiinia P, Kazemi B, Bandehpour M. Identification of different Theileria species (Theileria lestoquardi, Theileria ovis, and Theileria annulata) in naturally infected sheep using nested PCR–RFLP. Parasitol Res. 2011;108(4):837-43.
17. Chauhan H, Patel B, Bhagat A, Patel M, Patel S, Raval S, et al. Comparison of molecular and microscopic technique for detection of Theileria annulata from the field cases of cattle. Vet World. 2015;8(11):1370.
18. Naz S, Maqbool A, Ahmed S, Ashraf K, Ahmed N, Saeed K, et al. Prevalence of theileriosis in small ruminants in Lahore-Pakistan. J Vet Anim Sci. 2012;2:16-20.
19. Al-Hamidhi S, Elshafie EI, Yaghfoori S, Morrison WI, Johnson EH, Babiker HA. A comparative study of single Theileria lestoquardi and mixed infections with Theileria ovis. Parasit Vectors. 2021;14(1):1-10.
20. Heydarpour BM, Khazraeinia P, Hadadzadeh H, Kazemi B. Identification of Theileria species in sheep in the eastern half of Iran using nested PCR-RFLP and microscopic techniques. 2010.
21. Hegab AA, Fahmy M, Mahdy OA, Wahba A. Parasitological and molecular identification of Theileria Species by PCR-RFLP Method in Sheep, Egypt. Int J Adv Res Biol Sci. 2016;3(7):48-55.
22. Ceylan O, Byamukama B, Ceylan C, Galon EM, Liu M, Masatani T, et al. Tick-borne hemoparasites of sheep: a molecular research in Turkey. Pathogens. 2021;10(2):162.
23. Renneker S, Abdo J, Bakheit M, Kullmann B, Beyer D, Ahmed J, et al. Coinfection of sheep with Anaplasma, Theileria and Babesia species in the Kurdistan Region, Iraq. Transbound Emerg Dis. 2013;60:113-8.
24. Khayyat S, Gilder AA. Ovine piroplasmoses in Iraq. Trans R Soc Trop Med Hyg. 1947;41(1).
25. Erdemir A, Aktas M, Dumanli N, Turgut-Balik D. Isolation, cloning and sequence analysis of the lactate dehydrogenase gene from Theileria annulata may lead to design of new anti Theilerial drugs. Vet Med. 2012;57(10):559-67.