

CONVENTIONAL AND MOLECULAR DETECTION OF *BABESIA CABALLI* AND *THEILERIAEQUI* PARASITES IN INFECTED CAMELS IN SOUTH OF IRAQ

Hussein Jabar Jasim* Ghazi Yahgoob Azzal**

Rasha Munther Othman***

Department of Veterinary Microbiology and Parasitology, College of Veterinary
Medicine, University of Basra, Basra, Iraq.

(Received 7 September 2015 , Accepted 4 October 2015)

Key words: Babesia, Theileria, Camels.

ABSTRACT

The aim of this study was to identify and diagnosis of piroplasmosis (*Babesiacaballi* and *Theileriaequi*) in camels, based on microscopic examination and molecular diagnosis by using a polymerase chain reaction technique (PCR). The period of study was extended from August 2014 to March 2015, a total of 241 blood samples were collected from camels of both sexes, with different ages. Blood samples were collected from massacres and herds scattered in the provinces of Al Muthanna and Basra. The microscopic examination revealed two types of blood parasites (*Babesiaspp* and *Theileriaspp*) in camels at percentage of 9.95% and 5.8%, respectively. On the other hand the molecular diagnosis based on PCR was showed high specificity and sensitivity for the diagnosis of *B. caballi* and *T. equi*. Moreover the percentage of infection by *B. caballi* was recorded using two specific primers Bec-UF2/Cab-R and TBM/BCR to be 39.47% and 31.57% respectively. Whereas the percentage of *T. equi* infection was also recorded by using two specific primers Bec-UF2/Equi-R and TBM /Equi-R to be 23.68 % for both primers.

INTRODUCTION

Camel is physiological and anatomically adapted to survive harsh condition and play an important role in human's life how lived in desert and semi-desert and even in irrigate land. Camels like other domestics animals also exhibit to various pathogenic, infectious agents and disease. The ticks play an important role in the transmission of many different pathogenic organisms, but the most important are protozoan

pathogens. Diseases caused by protozoan parasites in ruminant are known as piroplasmosis include Theileriosis and Babesiosis(1).

Piroplasmosis is a tick borne haemoprotozoan disease caused by intra- erythrocytic protozoan parasites which include *Babesiacaballi* and *Theileriaequi* and Infects horses, mules, zebra, dogs and camels(2,3,4).Both *B. caballi* and *T.equi* are transmitted by several tick species belongto the genera *Hyalomma*, *Rhipicephalus*and*Dermacentor*(5).On other hand the piroplasmosis is one of the most important problems of the livestock industry in developing countries due to their responsible for significant economic losses to the livestock (6). The first cases of camels infected by *Babesiaspp* and *Theileriaspp* were recorded in Egypt (7).

The microscopic examination of blood smear stained with Giemsa was used for conventional diagnosis of *Babesiaspp*and *Theileriaspp*in infected animals during the acute phase of infection (8). However, this method is low sensitivity and efficiency to detect the carrier animals, due to parasitaemia may be low during the chronic phase of infection(9).Therefore a molecular technique is necessary for the diagnosis of piroplasms (*B.caballi* and *T. equi*), especially polymerase chain reaction which had been developed in order to overcome the problems faced with conventional and serological techniques, In addition PCR is reliable method for diagnosis and epidemiological studies, as well as permits the identification of genetic variants and cryptic species (4).Moreover, The 18S rRNA gene is most suitable genetic markers for diagnosis and phylogenetic studies of piroplasmida, due to it is low substitution, constraint and conserved function occurrence in multiple copies(10).The aim of this study was to identify and diagnosis the *B.caballi* and *T.equi* in the infected camels based on light microscope and PCR technique.

MATERIALS AND METHODS

Animals and Samples Collection

The study was started from August 2014 to March 2015.A totalof 241camels blood samples were collected from massacres of Samawah andRumathiaatMuthanna province and from Herds scattered (flocks) in south west of Basra province at Al-zubiar district. Animals are of 8 monthsto 7 years old andof both sexes (182 males and 59 females). Blood sample was drawn from the jugular vein and occasionally the sample was collected from tip of ear vein. Blood samples were drawn by10 ml sterile

syringe from infected camels. Six ml of blood was placed in two labeled sterile tubes containing anti-coagulant (EDTA). All blood collection tubes were inverted gently five times for directed mixing after collection and before storage.

The first blood sample was stored immediately in freeze at -20°C. These samples were used for molecular diagnosis based on polymerase chain reaction (PCR). While the second sample was used directly for making the blood smear for diagnosis the parasites present in the blood of camels (11).

Parasitological Examination

The blood smear was performed according to method of (11). The light microscopy was used for determine the presence of hemoprotozoal parasites during the acute phase of the disease give the best results. Each slide was examined under light microscopy for 20 minute.

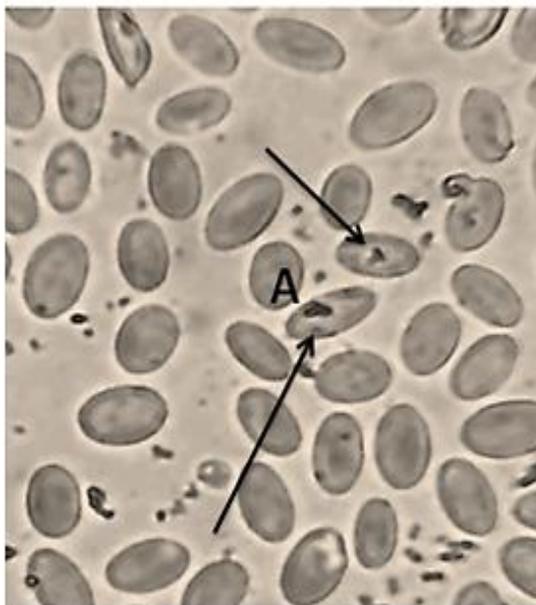
DNA Extraction and PCR Assay

The genomic DNA was extracted from the whole blood of the infected camels with piroplasmiasis by using (DNeasy Blood and Tissue Kit /QIAGEN / Germany). The first PCR reaction used to detect the *B.caballi* and *T. equi* parasites in the infected camels using specific primer (Bec-UF2; 5'-TCG AAG ACG ATC AGA TAC CGT CG-3'), and two reverse primer (Cab-R-5'-CTC GTT CAT GAT TTA GAA TTG CT-3'), and (Equi-R; 5'-TGC CTT AAA CTT CCT TGC GAT-3'), which amplify 540 bp and 392 bp long region of 18rRNA from both *B.caballi* and *T. equi* respectively (12). The PCR reactions were conducted in a total volume of 25 µl, consisted of 12.5 µl of Green master mix (Promega / USA), 5 µl of genomic DNA, 1 µl of each primer and 5.5 µl of nucleus free water. Amplification condition was obtained with an initial denaturation step at 96°C for 10 min followed by 40 cycle each at 96°C for 1 min, 60.5°C for 1 min and 72°C for 1 min, with final extension period of 10 min at 72°C (12). While the second PCR reaction used to distinguish between, *B. caballi* and *T. equi* using specific primer (TBM-F-5'-CTT CAGCACCTTGAGAGAAATC-3'), and two reverse primer (BCR-R-5'-GAT TCGTCGGTTTT GCCTTGG-3'), and (Equi-R - 5'-TGC CTT AAA CTT CCT TGC GAT-3'), which amplified 650 bp and 360 bp region of 18rRNA from both *B.caballi* and *T. equi* respectively (13). The PCR program performed of 94°C at 5 min followed by 40 cycle of 94°C for 1 min, 63°C for 1 min and 72°C for min, this was followed by a

final extension step at 72 °C for 10min (13). All results of the PCR were analysis after amplification stage. Five µl from amplification samples were directly loaded in a 1.5 % agarose gel electrophoresis and the products were visualized by UV trans illuminator.

RESULTS

Microscopic Examination The microscopic examination was performed by light microscope through laboratory examination to blood smears by using thin blood films staining and these examinediagnosis two genera of blood parasite which include*Babesia*spp and *Theileria*spp. Twenty four from 241 examined camels were found infection with *Babesia*spp at infection rate of9.95%based on Giemsa stained blood smears,moreover the *Babesia*spp was identified as large pear shape and arranged in pairs with acute or wide angles near the margin of the infectedRBCs figure (1).Onthe other hand the microscopic examination revealed that only 14 from 241 examined camels were found infectedwith *Theileria*sppat infection rate of 5.8%. *Theileria*spp were detected in RBCs withtwo forms include;rod and comma shape as in figure (2).



Figure(1) Blood smear of camel infected with *Babesia*spp (A: large pear shape and arranged in pair inside RBCs)(Giemsa stain, 1000x)

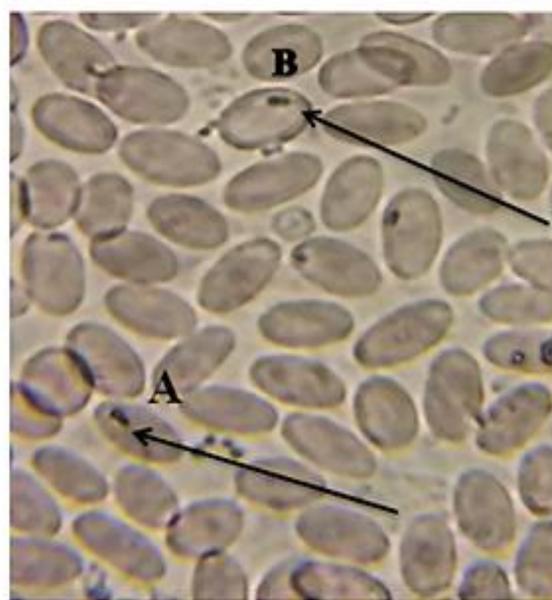


Figure (2) Blood smear of camels infected with *Theileria* spp (A: coma shape, B: rod shape inside RBCs) (Giemsa stain, 1000x)

Results of Molecular Diagnosis

The PCR technique was performed by using four specific primers to diagnosis both *Babesiacaballi* and *Theileriaequi* in the infected camels. The first PCR reaction aimed to confirm the *B.caballi* infection in camels by using specific primer (Bec-UF2 , Cab-R) for amplifying 540 bp region of 18S rRNA . Results were revealed that 15 (39.47%) out of 38 camels were positive to *B. caballi* in PCR technique as shown in figure (3). On the other hand the second primer (Bec-UF2, Equi-R) was chosen to diagnosis the *T. equi* in infected animals by amplifying 392 bp region of 18S rRNA. The result of PCR shows that 9 (23.68%) out of 38 camels were positive to *T.equi* figure (4). Additionally the third and fourth specific primers (TBM, BCR, Equi-R) were used to distinguished between *B. caballi* and *T. equi* through amplify the 650bp and 360bp region of 18S rRNA respectively as in figures (5,6) .ThePCR result was shown only 12 (31.57%) and 9 (23.68%) from 38 camels were positive to *B.caballi* and *T. equi* respectively .

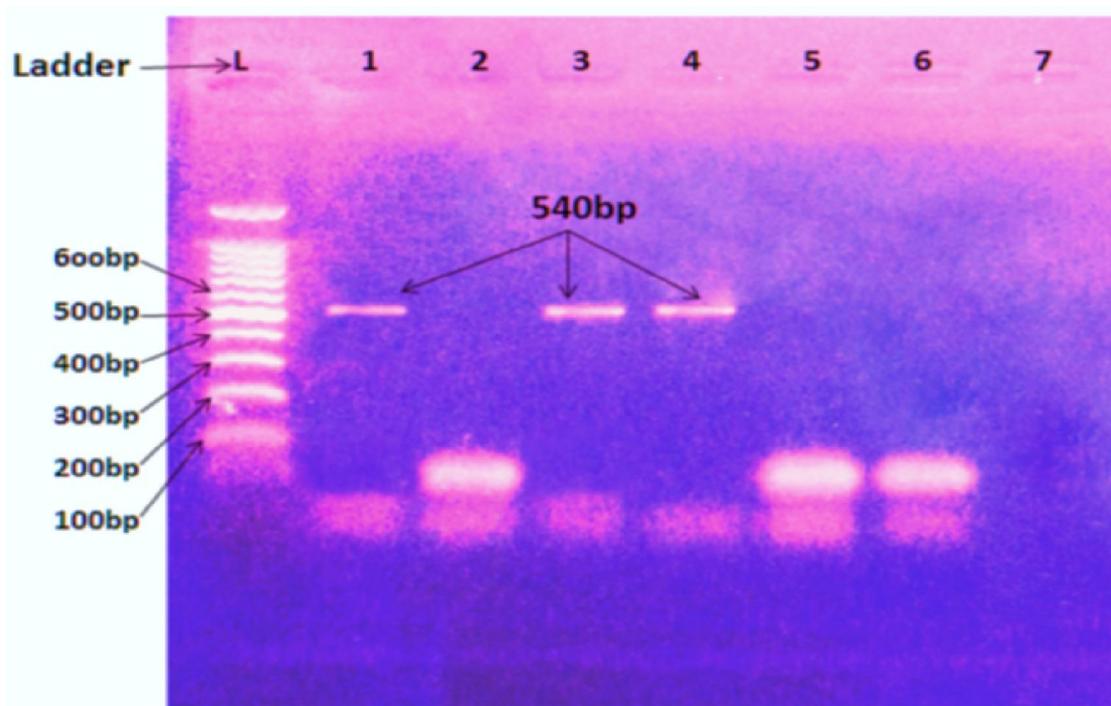


Figure 3.Agarose gel electrophoresis of amplified DNA from *Babesiacaballi* by using primers (Bec-UF2 , Cab-R) region of 18S rRNA. Lane L: DNA ladder. Lanes 1, 3, 4: positive product of (Bec-UF2, Cab-R) region of 18S rRNA

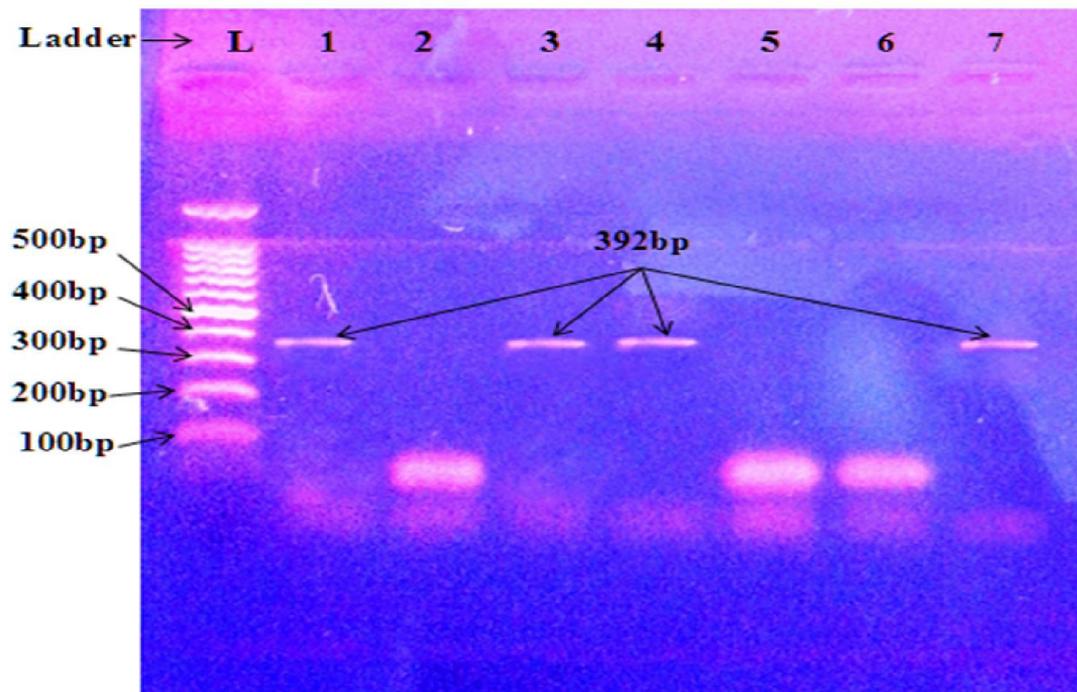


Figure 4 . Agarose gel electrophoresis of amplified DNA from *Theileriaequi* by using primers(Bec-UF2 , Equi-R) region of 18SrRNA . Lane L: DNA ladder . Lanes 1,3,4,7:positive product of (Bec-UF2 , Equi-R) region of 18SrRNA

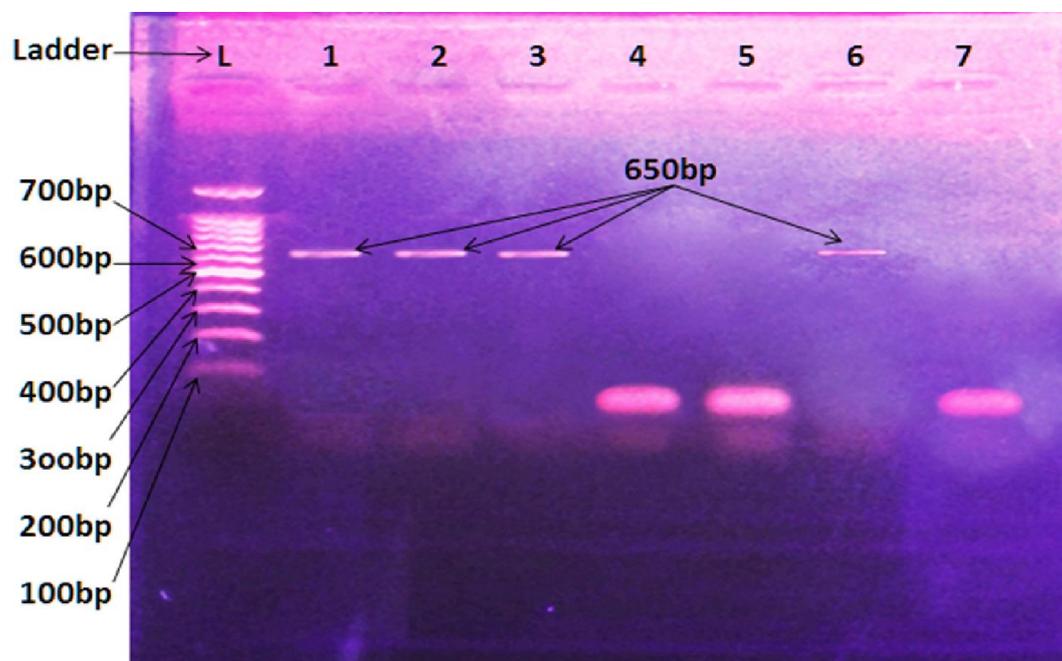


Figure 5 . Agarose gel electrophoresis of amplified DNA from *Babesia caballi* by using primers (TBM , BCR) region of 18SrRNA . Lane L: DNA ladder. Lanes 1,2,3,6:positive product of TBM , BCR) region of 18SrRNA

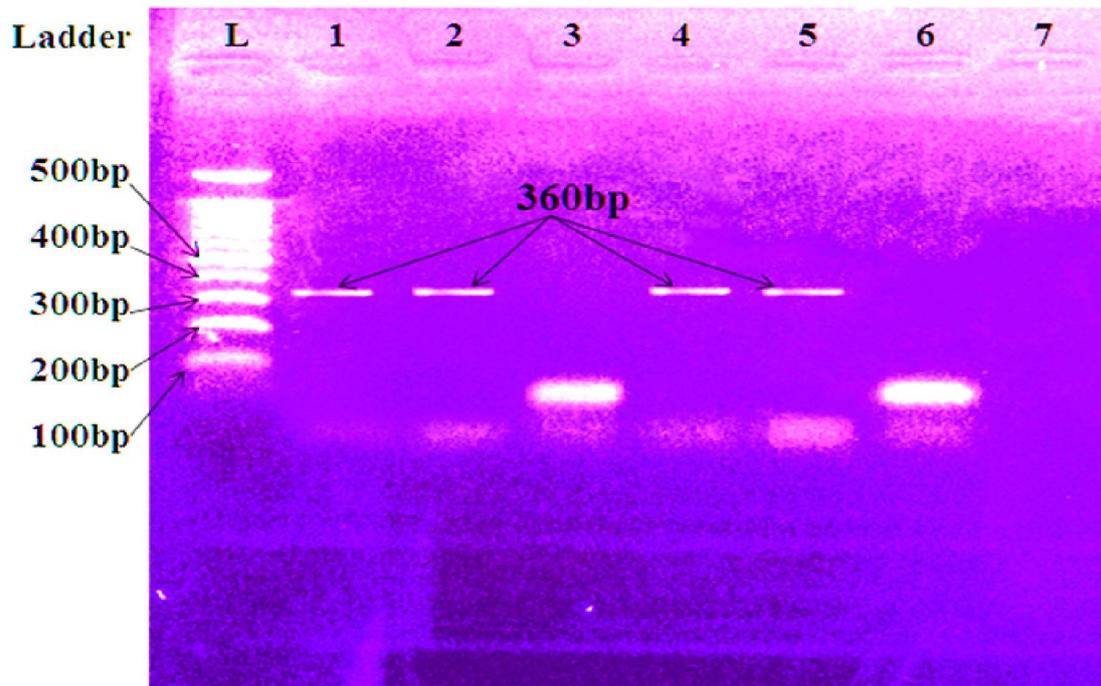


Figure 6. Agarose gel electrophoresis of amplified DNA from *Theileriaequi* using primers (TBM , Equi-R) region of 18SrRNA.Lane L: DNA ladder . Lanes 1,2,4,5 :positive product of(TBM , Equi-R) region of 18SrRNA

DISCUSSION

The results of the present study revealed that the local camels were infected with *Babesia*spp and *Theileria*spp and this fact confirms that the disease is widespread and affects all animal species including camels(3,7). The spread of piroplasmosis may occurs due to the specificity of *Babesia*spp and *Theileria*spp for the host , according to the present results this concept is probably lower than to be consider, which is supported by the detection of *Babesiacaballi* and *Theileriaequi* in other animals species like dogs and camels(3, 14). The possible explanation of this results is the ability of the ticks to meal among the camels and other animals(15).On the other hand the present study is also consider the first study in Iraq to distinguished between the*Babesia*spp and *Theileria*spp in camels based on molecular technique.

In the current study, the microscopic examination confirmed that *Babesia*spp and*Theileria*sppwas infected the camels with morbidity rate of 9.95% and 5.8%

respectively. Moreover the microscopic examination of the blood smear revealed that the *Babesia*spp and *Theileria*spp appear in many shapes inside the erythrocyte and these finding are agreement with other studies (7,16,17),and disagreement with (3),who did not recorded any infection with *Babesia*spp and *Theileria*spp based on the microscopic examination, butthey recorded 6 camels were found to have infected with *B. caballi*and *T.equi* based on polymerase chain reaction in the same study, the explanation of this discrepancy is attributed to the low sensitivity of the microscopic examination when compare with PCR technique,due to the limitation of microscopic examination ,the morphologically similar species cannot be distinguished (18).In addition, the accurate diagnosis is dependent on the experience of the microscopies (19). On the other hand the microscopic examination cannot be used to detect the chronic infection in the animals due to low parasitemia(20).Furthermore, the identification of *Babesia* and /or *Theileria* species based on microscopic examination can be difficult due to the development stage.When piroplasmids infected different vertebrate hosts, they appear changes in form and size due to the pleomorphism(21).

Recently the most common approach currently applied for the detection and distinguished of many species of *Babesia*and *Theileria* is polymerase chain reaction (PCR). On the other hand the 18S rRNA gene consider the most appropriate genetic marker for both analysis and phylogenetic studies of piroplasmids attributed to the constancy and stability of the ribosomal RNA genes makes them a prominent target for species variation (10). In this study 38 camels blood samples were subjected to molecular analysis to detect 18S rRNA genes specified for *Babesia*spp and *Theileria*spp. Only 15/38 (39.47%) of camels blood samples were found to be infected with *Babesiacaballi* which harbor 18S rRNA (Bec-UF2/Cab-R) gene and 12/38 (31.57%) of camels infected by *Babesiacaballi* which haven 18S rRNA (TBM/BCR) gene .In addition to 9/38 (23.68%) of camels blood samples were found to be infected with *Theileriaequi* which haven18S rRNA (Bec-UF2/Equi-R) gene and 18S rRNA (TBM /Equi-R) gene .These finding are agreement with results obtained by previous studies(4,22), and disagreement with (13).

The primer nucleotides fragment which was chosen in this study was previously used by (4, 22) to identification and distinguish between *B. caballi* and *T. equi* in the horses. In the present study the reason to choose these sets of primers was attributed to the common idea of grazing as well as the nature of local camels live in a direct or indirect contact with horses, donkey and dogs. Correspondingly, the detection of *B.*

caballi and *T.equi* in the local camels that live in the desert and away from the equide, possibly attribute to the transfer of animals from one location to another or by trading the herd such as by buying and selling of animals (3). Moreover, the dogs play an important role in transfer of *B.caballi* and *T.equi* infection to a camel, which is supported by the detection of equine piroplasms (*T.equi* and *B. caballi*) in dogs (2, 14). On the other hand, the ticks play an important role in the transmission of piroplasmids infection among the camels and other animals in fact, several ticks species belong to genera *Hyalomma* and *Rhipicephalus* invade both camels and horse (23). The present study concluded that the use of molecular PCR method to investigate and distinguish between *Babesia* spp and *Theileria* spp will enable us and facilitate the further identification of infected animals and/or asymptomatic carrier animals that could not be noticed by conventional methods.

Acknowledgement

The authors are grateful to Departments of Veterinary Microbiology and Parasitology, College of Veterinary Medicine, Al-Basra University- Iraq for providing the facilities.

الكشف التقليدي والجزيئي لطفيليات *Babesiacaballi* و *Theileriaequi* في الإبل المصابة في جنوب العراق

حسين جبار جاسم * غازي يعقوب عزال ** رشا منذر عثمان ***
 فرع الاحياء المجهرية والطفليات، كلية الطب البيطري، جامعة البصرة، البصرة

الخلاصة

هدفت هذه الدراسة كشف وتشخيص داء الكمثرات (*Theileriaequi* و *Babesiacaballi*) في الجمال، على أساس الفحص المجهرى والتشخيص الجزيئى باستخدام تقنيته (PCR). امتدت فترة الدراسة من اب ٢٠١٤ الى آذار ٢٠١٥. جمعت ٢٤١ عينة دم من الجمال، من مختلف الأعمار ومن كلا الجنسين، عينات الدم كانت تجمع من المجازر و القطعان المنتشرة في محافظتي المثنى والبصرة. كشف الفحص المجهرى عن تواجد نوعين من الطفيليات الدموية (*Babesia* spp و *Theileria* spp) في الجمال بنسبة 9.95%، 5.8% على التوالي. فضلا عن ذلك كشف التشخيص الجزيئى بالاعتماد على تقنيته التفاعل المتسلسل البوليمرات (PCR) عن ظهور تخصصي هو حساسية عالية لتشخيص كل من *T.equi* و *B. caballi*. علاوة على ذلك، سجلت نسبة الاصابة *B.caballi* باستخدام نوعين من البادئات الخاصة (Bec-UF2/Cab-R)، (TBM/BCR) كانت 39.47%، 31.57% على التوالي. في حين ان نسبة الاصابة المسجلة *T.equi* باستخدام نوعين من البادئات الخاصة (TBM /Equi-R)، (Bec-UF2/Equi-R) كانت 23.68% لكلا البادئين.

REFERENCES

1. Makala , L.H.; Mangani, P.; Fujisaki, K. and Nagasawa , H. (2003). The current status of major tick borne diseases in Zambia, Vet.Res., 34, 27-45.
2. Beck , R.; Vojta , L.; Mrljak , V.; Marinculi', c. A.; Beck , A.; Zivicnjak , T. and Caccio , S.M. (2009). Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. International Journal for Parasitology., 39 :843–848.
3. Qablan ,M.A.; Sloboda ,M .; Jiruka , M.; Obornik , M.; Dwairi,S. ; Amer, Z.S.; Horn ,P. ; Lukes ,J. and Modry , D. (2012). Quest for the piroplasms in camels : identification of *Theileria equi* and *Babesia caballi* in Jordanian dromedaries by PCR .Vet.parasitol.,186: 456-460.
4. Qablan ,M.A.; Obornik ,M. ; Petzelkova, K. ; Sloboda, M. ;Shudiefat, M.; Horin,P. ; Lukes, J. and Modry ,D.(2013). Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids : epidemiology and genetic diversity .parasitology., 140(9) :1096 -1103.
5. De Waal , D. T. (1992). Equine piroplasmiasis: a review. Br Vet J., 148(1): 6–14.
6. Aktas, M.; Altay, K.; Ozubek, S.; and Dumanli , N. (2012). A survey of ixodid ticks feeding on cattle and prevalence of tick-borne pathogens in the Black Sea region of Turkey. Vet. Parasitol., 187 (3-4):567-571.
7. Abd-Elmaleck, B.S., Abed, G.H. and Mandourt, A.M. (2014). Some Protozoan Parasites Infecting Blood of Camels (*Camelus dromedarius*) at Assiut Locality, Upper Egypt. J. Bacteriol . Parasitol., 5: 184.
8. Shkap, V. ; Cohen, I ; Leibovitz, B ; Savitsky, Pipano, E ; Avni, G., Shofer, S ; Giger, U ; Kappmeyer, L. and Knowles, D. (1998). Sero -prevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Vet. Parasitol.,76:251–259.
9. Friedhoff , K. and Bose, R. (1994). Recent developments in diagnostics of some tick-borne diseases. In: Uilenberg, G., Permin, A., Hansen, J.W. (Eds.), Use of Applicable Biotechnological Methods for Diagnosing Haemo- parasites. Proceedings of the Expert Consultation, Merida, Mexico, October 4–6, 1993. Food and Agriculture Organisation of the United Nations (FAO), Rome, Italy, pp. 46–57.

10. Hunfeld ,K. P.; Hildebrandt , A. and Gray, J. S. (2008). Babesiosis: recent insights into an ancient disease. *Int. J. Parasitol.*, 38:1219 –1237.
11. Chaudhri , S.S. and Gupta, S.K.(2003). *Manual of General Veterinary Parasitology*. 1st edition . department of Vet. Parasitology College of Vet. Sci. Haryana Agricultural University India.Pp:46-47.
12. Alhassan , A.; Pumidonming , W.; Okamura , M.; Hirata, H.; Battsetseg , B.; Fujisaki, K.; Yokoyama , N. and Igarashi, I. (2005). Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesiacaballi* and *Babesiaequi* in horse blood .*Vet Parasitol.*, 129 :43–49.
13. Sloboda , M.; Jirků , M.; Lukešová , D.; Qablan , M. A.; Batsukh ,Z.; Fiala , I.; Hořín , P.; Modrý , M. and Lukeš , J. (2011). A survey of piroplasmids in horses and Bactrian camels in North-Eastern Mongolia. *Vet. Parasitol.*,179:246–249.
14. Fritz, D. (2010) .A PCR study of piroplasms in 166 dogs and 111 horses in France (March 2006 to March 2008).*Parasitol. Res.* 106, 1339-1342.
15. Nazifi,S.;Tamadon,A.;Behzadi,M.A.;Haddadi,S.;Raayat-Jahromi, A.R.(2011). One-humped camels (*Camelus dromedaries*) hard ticks infestation in Qeshm Island, Iran . *Vet. Res. Forum.*(2) :135-138.
16. Ismeal , A.B.; Swelum , A.A.;Khalaf ,A.F. and Abouheif, M.A.(2014).Clinical haemtological and biochemical alterations association with an outbreak of theileriosis in dromedaries (*Camelus dromedaries*) in Saudi Arabia . *Pak ,Vet J.*, 34(2): 209-213.
17. Swelum ,A.A.; Ismeal ,A.B.; Khalaf ,A.F. and Abouheif ,M.A.(2014). Clinical and laboratory findings associated with naturally occurring babesiosis in dromedary . Saudi Arabia . *Bull. Vet. Inst .Pulawy .*, 58 :229-233.
18. Conrad, P.A.; Thomford , J. W.; Marsh, A.; Telford, S. R.; Anderson, J. F.; Spielman, A.; Sabin, E. A.; Yamane I. and Persing,D. H. (1992). Ribosomal DNA probe for differentiation of *Babesiamicroti* and *B. gibsoni* isolates. *J ClinMicrobiol.*, 30(5):1210-5.
19. Morgan, U. M .(2000). Detection and characterisation of parasites causing emerging zoonoses . *Int. J .Parasitol.*, 30:1407-1421.
20. Khamesipour , F .; Doosti, A.; Koohi , A.; Chehelgerdi ,M.; Mokhtari- Farsani , A.andChengula , A .A . (2015). Determination of the presence of

*Babesia*DNA in blood samples of cattle, camel and sheep in IRAN by PCR.
Arch. Biol. Sci Belgrade., 67 (1):83-90.

21. Homer, M.J.; Aguilar-Delfin, I.; Telford, S.R.; Krause, P.J. and Persing, D.H.(2000). Babesiosis. Clin. Microbiol. Rev., 13(3): 451-469.
22. Malekifard ,F.; Tavassoli ,M.; Yakhchali ,M. and Darvishzadeh, R.(2014). Detection of *Theileriaequi* and *Babesiacaballi* using microscopic and molecular methods in horses in suburb of Urmia, Iran . Vet. Res. Forum., 5 (2) 129 – 133.
23. Walker, J.B.; Keirans, J.E. and Horak, I.G. (Eds.), 2000. A Guide to the Brown Ticks of the World. Cambridge University Press, New York, p. 669.