SEOPREVALENCE AND MOLECULAR DETECTION OF *BABESIA* SPECIES IN CATTLE IN BAGHDAD CITY

Abdelrasol .KH.Saeed*, Mohammed.TH.Salih** Mohammed Sh. Jebur *

*Department of Medical laboratory Technology,College of Medical and Health technology,Middle Technical University,Baghdad,Iraq.
**Department of Parasitology,College of Veterinary Medicine,University of Baghdad,Baghdad,Iraq.

(Received 28 March 2017,Accepted 7 May 2017)

Keywords: *Babesia bovis,* Blood smear ,ELISA.

Correspond Author; mohabood24@gmail.com

ABSTRACT

Cross-sectional study carried in areas surrounding Baghdad province (Dora , Nahrawan, Tajy and Abu Graib)respectively. During June -December 2016, for detection of cattle *Babesiosis*. One hundred fifty (150) clinically healthy local cattle breeds of different ages, and from both sex were examined by routine blood smear examination, molecular detection of Babesiosis by Conventional polymerase chain reaction PCR and by ELISA test. Results revealed that the infective species of *Babesia* during this study were *B.bovis* and *B.bigemina* that had detected in 14 cases (9.33%) by traditional blood smear examination, compared with 15 cases (10%)with *Babesia bovis* by conventional PCR technique and 100 cases (66.6%)with *Babesia bigemina* were detected by using ELISA technique .It has been concluded that infection with *Babesia.bovis and Babesia bigemina* were more prominent and no other species were detected.

INTRODUCTION

Bovine Babesiosis or Piroplasmosis also called red water disease of cattle are protozoan parasitic diseases caused by Genus *Babesia* Order *Piroplasmida* Phylum *Apicomplexa.* (1).

Babesia bigemina and *Babesia bovis* are the most widely distributed species affecting cattle in most part of the world except some part of Europe were *Babesia divergens* are the most prevalent .(2).

Diagnosis of Babesiosis is same as for other hemoprotozoan infections were the conventional diagnostic methods are still depend on the demonstration of infective stages in the blood , by Giemsa stained blood smear were it consider the gold standard for diagnosis, beside detection of circulating antibodies in the serum of infected animals by serological test like Enzyme Linked Immunosorbent Assay (ELISA) (3), (4).More over Polymerase chain reaction(PCR) had been widely used for detection of

Babesia parasites owing to their high specificity and sensitivity (5),(6).Because of little studies concerning bovine babesiosis generally in Iraq and specially in Baghdad province as the capitol of Iraq ,except few studies in the south and the north of Iraq that used one or two diagnostic techniques, beside the current study aimed to sight a light on *Babesia* species in cattle of Baghdad.

MATERIALS AND METHODS

Collection of Blood samples

Blood sample were collected from 150 apparently healthy cattle from different parts of Baghdad Province (96 female and 54 male), with ages ranging between 1-10 years, during the period between June 2016 –December 2016.Blood were collected by jugular vein puncture by two type of vacutainer tube ,one coated with EDTA for PCR technique. The second tube without EDTA for serum collection for ELISA technique.

Direct Detection of Babesia species in blood sample

1-Standard Blood smear taken from ear veins staining with Giesa stain and examination were done according to(7).

2-Conventional PCR (nested) PCR technique were used according to (5) and manufacturer instructions (Inqaba Biotechnical Industries, Pretoria, South Africa).This procedures includes DNA extraction and primer design according to(Inqaba Biotechnical Industries, Pretoria, South Africa).

-DNA Extraction:

- The blood sample had mixed for 10 minutes at room temperature.
- A volume of 20 µl of Proteinase K (PK) had dispensed into a 1.5 ml micro-centrifuge tube.
- From each sample, 250 µl of blood was added to the tube containing the Proteinase K (PK) solution ,and briefly mixed.

- Cell lysis Buffer (CLD) had added in avolume of 200 µl into a tube. This tube has capped and mixed by vortexing for 10 seconds
- In water bath, tubes had incubated at 56 c˚ (optimal for Proteinase activity) for 10 minutes .
- While the blood sample had incubated , a ReliaPrep™ Binding Column had placed into empty collection tube.
- After tube removing from water bath incubation, a volume of 250 µl of binding Buffer (BBA) had added for each tubes. These tubes had capped and mixed by vortexing for 10 minutes.
- Tubes contents had added to the labeled ReliaPrep ™ Binding Column, there for the column has capped and placed in microcentrifuge.
- Centrifugation had conducted for 1 minute at maximum speed.
- Collection tubes containing the flow through had removed, and the liquid has discarded as hazardous waste.
- Binding columns had placed into a new collection tubes A volume of 500 µl of column wash solution (CWD) has added to the column and centrifuged for 3 minutes at maximum speed through that the flow has excluded.
- Washing steps had repeated twice for three washes.
- Columns had placed in a clean 1.5 ml microcentrifuge tubes.
- Volume of 200 µl of nuclease-free water had added to the column and centrifuged for 1 minute at maximum speed.
- Binding Column has discarded and the eluate has saved at -30 c˚. (8).

Determination of DNA Concentration and Purity:

Quantusflorometer used to detect the concentration of the extracted DNA in order to detect the quality of samples for downstream application . For 1 µl of DNA , 199 µl of diluted Quanty flour dye has mixed. There for, after incubation at room temperature , DNA concentration values had detected.(9).

Detection of Presence of *B.bigemina* and *B.bovis* :

Purified DNA samples had used to assess the specificity of Group 1 and Group 11 primers (Table 1), that described by (5).

Detection the presence of *B.bigemina* and *B.bovis* **,** from study blood samples , using Grpup 1 primer , PCR product has added into the second (nested) PCR mixture compromising similar composition of reagents as the first-round PCR except that the external primers had replaced with the nested PCR primers.

Table (1) : Primers Used for PCR Amplification

Name	Seq.	Tm	Size
B. bigemina PCR-F	CATCTAATTTCTCTCCATACCCCTCC	55	278
B. bigemina PCR-R	CCTCGGCTTCAACTCTGATGCCAAAG		
B. bigemina nPCR-F	CGCAAGCCCAGCACGCCCCGGTGC	55	170
B. bigemina nPCR-R	CCGACCTGGATAGGCTGTGTGATG		
B. bovis PCR-F	CACGAGGAAGGAACTACCGATGTTGA	55	360
B. bovis PCR-R	CCAAGGAGCTTCAACGTACGAGGTCA		
B. bovis nPCR-F	TCAACAAGGTACTCTATATGGCTACC	55	298
B. bovis nPCR-R	CTACCGAGCAGAACCTTCTTCACCAT		

Phylogenetic Analysis:

For phylogenetic study , genomic DNA of randomly selected field sample was amplified with Group11 nested PCR primers (Table 2) targeting gp45 and rap-1 fragments of *B.bigemina* and *B.bovis* , respectively (5).

10(ten) bovine samples has selected for *B.bigemina* and 10 (ten) for *B.bovis*.PCR mixture were prepared and randomly cycled, were described in following ,PCR generated fragments of 853 bp for *B.bigemina* and 1009 bp for *B.bovis.*

Table (2) Phylogenetic analysis of B.bovis and B.bigemina.

Name	Seq	Tm	Size
G2_*B. bigemina* PCR-F	GTGCTGCTTAATCGCACAAAC	55	963
G2_*B. bigemina* PCR-R	AAGATGCCTTCTTCGGTGATG		
G2_*B. bigemina* nPCR-F	CGGATCCTGTTATCGTTCCTG	55	853
G2_*B. bigemina* nPCR-R	GAAGTTACGCCTGGAGTTGG		
G2_*B. bovis* PCR-F	TCAGATTGTTCAAAGAGAGTGCATCC	55	1280
G2_*B. bovis* PCR-R	GTCTTCACCGTTGGAAGTAGTTGAGTC		
G2_*B. bovis* nPCR-F	CACGAGGAAGGAACTACCGATGTTGA	55	1009
G2_*B. bovis* nPCR-R	CCTTTGTAGGTTGGCCAACAGTTTCG		

Detection of Babesia species in purified DNA samples;

For the detection of the presence of *B.bovis* and *B.bigemina* in study DNA samples by using primers in table(1), PCR was performed according to manufacturer instructions (Inqaba Biotechnical Industries). Negative control reactions contained distilled water instead of template DNA.

PCR generated amplicons were analysed by electrophoresis in 1.5 % agarose gels stained by Biotium GelRed Acid Stain (Anatech Instruments,Johannesburg,South Africa) under UV illumination.

GeneRuler 1 kb plus DNA ladder were used as the standard molecular weight marker.

Indirect Detection of *Babesia* species in study serum samples:

Indirect ELISA technique were used according to (10), and manufacturer instructions (SVANOVA * Boehringer Ingelheim Svanova) Uppsala , Sweden.

SVANOVA *B.bigemina* Antibody test were used according to (11) and (12), kits for *B.bovis* were not available during this study due stop manufacturing them in most of laboratories.

Statistical Analysis:

Results statistical analysis were carried using SPSS –version 14,using t-test and chi-square test according to (13).

RESULTS AND DISCUSSIONS

Blood smear examination of (150) cattle of different age group and of both sex in Baghdad province revealed infection with ***Babesia*** species in (14) cattle ,with infection rate(9.33%). Figure(1),Table (3).

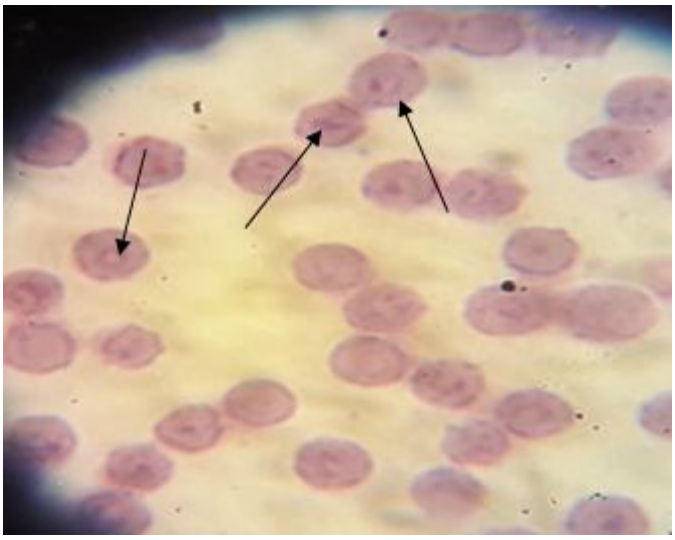


Figure (1) . Erythrocytic stages of ***Babesia*** *spp* in Giemsa stained blood smear pointed by arrows . **x 100.**

Table (3) . Prevalence rate of cattle Babesiosis in Baghdad province according to Blood smear examination.

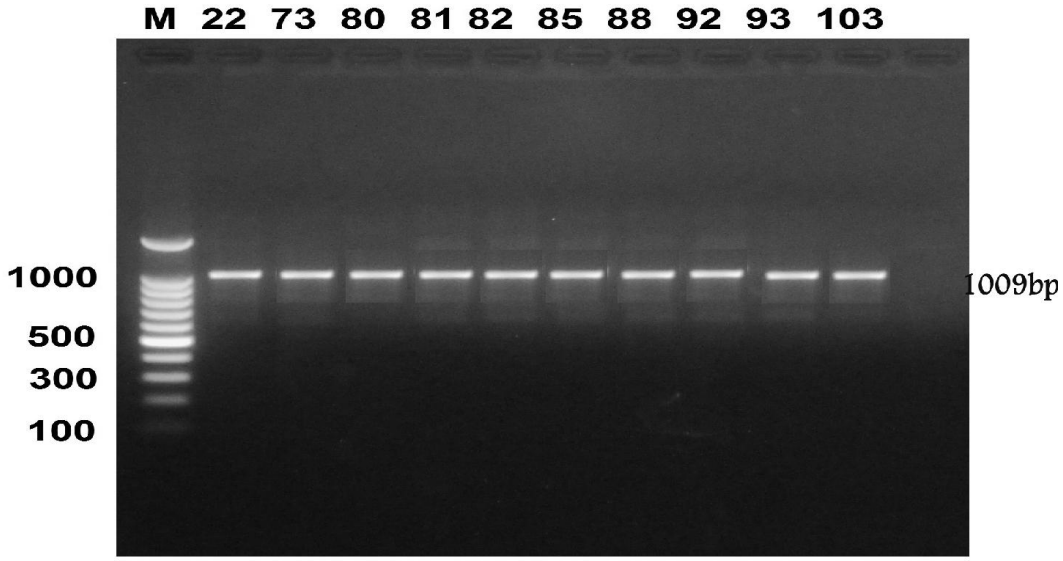
BLOOD Smear EXAMINATION	No	%
Negative	136	90.67
Positive	14	9.33
Total	150	100.00

Results were agreed with many studies records like that of (14)in Iraq 8.8% ,in Pakistan 7%, (15). in Egypt, 7.69% (16) and (17) in Ethiopia who recorded 4.4%. Results of this study were lower than that recorded by other researchers such as 24% by (18) in Pakistan , while (19) recorded 16.4% in western Iran. This may due to geographical differences and distribution of tick vector in the study areas. and differences in the resistance of local breed against infection with babesiosis in different study areas.

Among PCR, results of this study revealed that 10% of samples were infected with *B.bovis* without recorded any infection with *B.bigemina* as shown in table (4) . Figure (2).

Table (4) .Nested PCR results according to species of *Babesia.*

POLYMERASE CHAIN REACTION TEST		SEX						
		Females		Males		Total		
		No	%	No	%	No	%	P.V
B.bigemina	negative	96	100.00	54	100.00	150	100.00	
	positive	0	.00	0	.00	0	.00	
B.bovis	negative	90	93.75	45	83.33	135	90.00	0.041*
	positive	6	6.25	9	16.67	15	10.00	
	Total	**96**	**100.00**	**54**	**100.00**	**150**	**100.00**	



Figure(2);PCR results showing positive pool samples on gel at 1009 bp for *B.bovis*. Lane M(100-1000 bp DNA ladder) , Lane (22,73,80,……..103) positive PCR product amplified from study blood samples.

Results were not agreed with most of studies concerning molecular prevalence of cattle Babesiosis , like (20) in Iraq , and(18) in Pakistan who recorded the presence of both *B.bovis* and *B.bigemena* in their studies, although using different genes and different primers design may play important role in this differences.

66.6% of samples in ELISA technique gave positive results for detection of *B.bigemina* in study sample serum ,this may due to presence of antibodies againest this parasite in study samples due to presence of vector of that species of the parasite, or strong reaction of their antibodies, this result were in agreement with (4). Table (5).

Table (5) . Results of study samples PCR and ELISA Compartion.

	Reaction	No	%
ELIZA	Negative	50	33.3
	Positive	100	66.6
	Total	**150**	**100.00**
PCR	Negative	135	90.00
	Positive	15	10.00
	Total	**150**	**100.00**

χ^2=10.392 df =3 p= 0.016*

نسبة الانتشار المصلي والتشخيص الجزيئي للإصابة بداء الكمثريات Bovine babesiosis في الأبقار في محافظة بغداد.

عبد الرسول خيرالله سعيد الزبيدي* محمد ثابت صالح الزبيدي** محمد شمخي جبر الذهيبات*

*كلية التقنيات الصحية والطبية,بغداد . الجامعة التقنية الوسطى ,بغداد،العراق.
** فرع الطفيليات ،كلية الطب البيطري ، جامعة بغداد ،بغداد،العراق.

الخلاصة

دراسة مقطعية اجريت في محافظة بغداد خلال الفترة من حزيران-كانون الاول ٢٠١٦ للتحري المصلي والجزيئ عن الطفيلي المسبب لداء الكمثريات في الابقارBovine babesiosis في المناطق المحيطة بمدينة بغداد وهي مناطق الدورة ،النهروان، التاجي وابو غريب على التوالي. شملت الدراسة ١٥٠ من الابقار الاصحاح عيانيا من العرق المحلي من مختلف الاعمار ومن كلا الجنسين(٩٦ انثى و ٥٤ ذكر) وشمل الفحص الذي اجري على هذه الابقار اخذ لطخات(مسحات) دموية من الوريد الاذني الحافي وصبغه بصبغة الكمزا، وسحب نماذج الدم من الوريد الوداجي لغرض فحص (الدنا) بطريقة تتفاعل سلسلة البلمرة التقليدي conventional polymerase chain reaction. وكذلك فحص ELISA الاليزا للتحري عن الاجسام المضادة للطفيلي في نماذج المصول التي تم عزلها من جميع حيوانات الدراسة، اظهرت النتائج ان ١٤|١٥٠ مسحة دموية اظهرت اشكال متعددة من الطفيلي نوع *Babesia* أي نسبة اصابة مقدارها ٩.٣٣% ،مقارنة بنسبة اصابة ١٠%اي ١٥|١٥٠ حالة موجبة بالطفيلي *Babesia bovis* باستخدام التشخيص الجزيئي PCR ،و تم تسجيل ١٠٠ حالة موجبة بالنوع *bigemina* باستخدام تقنية الاليزا اي نسبة اصابة مقدارها ٦٦.٦%، وان النوعين *B bigemina* و *B bovis*هما النوعان السائدين في منطقة الدراسة،مما يتطلب مزيدا من الاهتمام باستخدام طرائق التشخيص الحديثة لحساسيتها وخصوصيتها في تشخيص الاصابة بطفيلي بالبابيزيا

REFERENCES

1-O.I.E. Bovine babesiosis in Terrestrial manual.Vol.1.Ch 2.4. Office international Des Epizooties,World Health Organization for Animal Health,Paris, France.p 1-15, 2010.

2-Bock,R.,Jackson,L.,Devos,A. and Jorgensen,W.(2004).Babesiosis of cattle.Parasitology. 129:247-269.

3-Goff WL, Johnson WC, Molloy JB, Jorgensen WK, Waldron SJ, Figueroa JV, Matthee O, Adams DS, McGuire TC, Pino I, Mosqueda J. Validation of a competitive enzyme-linked immunosorbent assay for detection of Babesia bigemina antibodies in cattle. Clinical and Vaccine Immunology. 2008 Sep 1;15(9):1316-21.

4- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990 Oct 5;215(3):403-410.

5 -Mtshali MS, Mtshali PS. Molecular diagnosis and phylogenetic analysis of Babesia bigemina and Babesia bovis hemoparasites from cattle in South Africa. BMC veterinary research. 2013 Aug 8;9(1):154

6-AbouLaila M, Yokoyama N, Igarashi I. Development and evaluation of two nested PCR assays for the detection of Babesia bovis from cattle blood. Veterinary parasitology. 2010 Aug 27;172(1):65-70.

7- Hamsho A, Tesfamarym G, Megersa G, Megersa M. A Cross-Sectional Study of Bovine Babesiosis in Teltele District, Borena Zone, Southern Ethiopia. J Veterinar Sci Technol. 2015;6(230):2.

8-Almeria,S.,Castella,J.,Ferrer,D.,Ortuno,A.,Estrade,-Pena,A.,and Gutierrez,J.F.(2001).Bovine piroplasma in Minorca(Baleric island , Spain): a compartion of PCR-based and light microscopy detection.Vet.parasitol.99:249-259.

9-Fisher,TG.,McElwain,TF.,Palmer,GH:Molecular basis for variable expression

of merozoites surface antigen gp45 among American isolates of *Babesia bigemina*. Infect immune.2001,69:3782-3790.

10-Terkawi MA, Thekisoe OM, Katsande C, Latif AA, Mans BJ, Matthee O, Mkize N, Mabogoane N, Marais F, Yokoyama N, Xuan X. Serological survey of Babesia

bovis and Babesia bigemina in cattle in South Africa. Veterinary parasitology. 2011 Dec 15;182(2):337-42.

11-Tebele,N.1996: characterization of DNA encoding a 200 KD,a polypeptide of *Babesia bigemina* and generation of a recombinant antigen for detection of antibodies in an enzyme linked Immunosorbent assay. PhD thesis.Brunel University Uxbridge , United Kingdom.

12-Bono,M.F.,Mangold,A.J.,Bravalle,M.E.,Velntini,B.S.,Thompson,C.S.,Wilkowsky,

S.E.,Echaide,I.E.,Far,M.D. and Torioni De Echaide,S.M.(2008). Efficiency of a recombinant MSA-2s-based ELISA to establish the persistence of antibodies in cattle vaccinated with *Babesia bovis.*Vet.Parasitol.157:203-210 .

13-Snedecor GW CWG. Statistical methods .7th ed.Iowa.The state University Press American:1989.

14-Omima Ibrahim, Zaid Taha and Salih Jassim (2012).Prevalence of *Babesia bovis* in cattle in Tikreet city and its surrounding ,with heamatological study.Tikrit Journal of Pure Science:17(2) 2012.

15-Boonchit S.,Xuan X.,Yokoyama N., and Goff W.L. Improved enzyme-linked Immunosorbent assay using C terminal truncated recombinant antigens of *B.bovis* rhoptry associated protein 1 for detection of specific antibodies.J.Clin.Microbiol.2006. 42:1601-1604.

16- Salm, F.F., E.E. Younis, N.M. Hegazy and A. El-Sawalhy, 2011.Epidemiological Studies on Bovine Babesiosis.Bull.Anim.Hlth.Prod. Afr., 59:169-177.

17- Fethu Lemma, Adugna Girma and Dersha Demam . Advances in Biological Research 10 (1): 37-42 , 2016

18- Saad F, Khaisroon M, Khan K, ul Akbar N. Prevalence and Molecular Detection of Babesiosis in the Slaughter Animals of Peshawar Khyber Pakhunkhawa Pakistan. Int. J. Curr. Microbiol. App. Sci. 2015;4(8):1030-1036.

19-Fakhar,M.,A.Haji hasani,S.Maroufi,H.Alizadeh,H.Shirzad,F.Piri and A.S.Pagheh. An epidemiological survey on bovine and ovine babesiosis in Kurdistan province, Western Iran.Trop.Anim.Hlth.Prod.,2012.44:319-322.

20- Khawla Hussein Sabbar Al-Thabhawi (2015) Molecular Detection of *Babesia bovis* and *Babesia bigemina* in cattle in Al-Qadisiyah Province.
Msc thesis .College of Veterinary Medicine/University of Al –Qadisiyah.