Assiut Vet. Med. J. Vol. 39 No. 78, July

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USING OF RIBOSOMAL - DNA BASED PCR METHOD FOR SENSITIVE DETECTION OF CARRIER AND MILD BABESIAL INFECTIONS AMONG CATTLE IN EGYPT

(With One Table and 3 Figures)

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(Received at 1/6/1998)

استخدام ريبوزومل الحمض النووى (دى.إن.إيه) المعتمد على طريقة الإكثار (بى سى ار) للكشف عن العدوى بالبابيزيا فى الأبقار ذات الإصابه الطفيفه و الحامله للمرض فى مصر

جمال حلمى سالم

تم استخدام اختبار اكثار الحمض النووى (بى سى ار) فى تشخيص طفيل البابيزيا بنوعيه فى مصر وكذلك للتفريق السريع لعترات البابيزيا وذلك باستخدام طقمين من بوادئ اختبار (بى سى ار). وقد استخدم ايضا مجسات متخصصه لكل طفيل على حده . وتم تصميمهما جميعا معتمده على التتبعات الخاصه بنسخه الحمض النووىالديوكسي ريبوزى حامل شفرة الجزئ الصغير لحمض الريبوزللبابيزيا بوفس وباجيمينا (عترة المكسيك) كانت النتائج كالأتى: تم اكثار نفس الجزء من الحمض النووى لعترات البابيزيا فى مصر فى الضوابط بنفس الحجم بالمقارنه مع العترات المرجعيه من البابيزيا (عتره المكسيك) . وفى تجربه اخرى مكن هذا الإختبار من الكشف السريع بنجاح عن اقل تركيز من الحمض النووى لكل طفيل على حده وظهر نفس الجزء من الحمض النووى ولكن بكثافه متدرجه .وجد ان هذا الإختبار قادر عل اكتشاف ما يقرب من ١٥ طفيل من جنس بايجيمنا و ٢٠٠ طفيل من جنس بوفس فى١ مللى دم .وتم التاكد من جميع اختبارات اكثار الحمض النووى باجراء اختبار التهجين مع مجسات الحمض النووى الديوكسى ريبوزى المتخصصه الغير مشعه المحضره من التتابعات الخاصه ببابيزيا بوفس و بايجيمنا . وقد دلت النتائج على انه باستخدام اختبار اكثار الحمض النووى وجد ان ٣٠% من العينات ايجابيه لطفيل بابيزيا بوفس و ٤,٩% بطفيل بابيزيا بايجيمنا و ٥, % كانت مصابه بالطفيلين معا . وكذلك وجد ان ٨, ١ % من العينات ايجابيه بالفحص المجهرى ولكن لم نستطيع تميز العتره الموجوده والتى بالفعل امكن التعرف عليها باستخدام ال بى سى ار. وقد دلت النتائج على انه باستخدام هذا الإختبار يمكن

التفريق بين العترات المختلفه لطفيل البابيزيا وامكانيه تطبيقه كاختبار ذو قيمـــه فـــى الكشـــف بتخصص عن طفيل البابيزيا فى الحيوانات الحامله للمرض فى مصر.

SUMMARY

Two PCR primer sets and two oligonucleotide probes verified from the sequence of transcription units of DNA (ribosomal DNA, rDNA) encoding the small Subunit ribosomal RNA (SSr-RNA)genes of *B. bovis* and *B.bigemina*,Mexico strain (one set /each) were used to amplify a portion of these genes from both species in Egyptian cattle. The expected fragments by Polymerase Chain Reaction (PCR) were visualized and measured as 275 base pair (bp) and 175 (bP) corresponding to *B. bovis* and *B. bigemina* respectively. The amplified products were confirmed by Southern blot hybridization with nonradioactive (NR) species specific oligonucleotide probe. The sensitivities of these methods were 200 parasite /ml. blood in *B.bovis* and 15 parasites/ml. of blood in *B bigemina.* By the PCR method .30% of carrier cattle were infected with *B. bovis* and 40.9 % with *B.bigemina* as well as 4.5 % showed mixed infection, while with microscopical examination of Giemsa stained blood smears only 1.8% were detected. Cattle which were positive microscopically revealed the fragment of *B.bigemina* by PCR. This method provides a useful diagnostic tool for rapid detecting and testing the efficacies of drugs and vaccination used .

Key words: *Cattle – Babesia -- Detection – PCR.*

INTRODUCTION

Bovine babesiosis is a tick- borne disease found worldwide, caused by different species of the genus *Babesia* (McCrosker, 1981). Recovered animals may sustain a subclinical infection which is microscopically undetectable. These carrier animals, serve as reservoirs for infections in the herd and continue to infect the tick vector (Mahoney, 1969). Currently, detection of babesia infection in carrier animal is done either by examination of blood smears with low sensitivites and time consuming (Todorovic, 1975) or by serological methods with occasional interspecies cross reaction and cannot differentiate between post exposure and present infection (Morzaria et al.,1992). Nucleic acid probes for *B. bovis* and *B. bigemina* have been developed from repetitive genomic Deoxyribonucleic acid (DNA) by numerous investigators

(Buening et al., 1990 and Figueroa et al., 1993). These probes generally detect a level of parasitemia lower than that detected by light microscopy but they are not sufficiently sensitive for reliable detection of the carrier state (Fahrimal et al.,1992).

To increase this sensitivity, the target of native DNA or Ribonucleic acid (RNA) of the organism can be amplified by Polymerase Chain Reaction ; PCR (Saiki et al., 1988).

Ribosomal RNA (rRNA) offers an alternative target for detecting parasites in a host even at very low levels of infection, because rRNA is the most abundant cellular macromolecule. This facilitates the development of sensitive detection assays (Waters and McCutchan, 1990). Recently, complete nucleotide sequence of the small subunit ribosomal RNA (SSrRNA) genes of *B. bigemina* (Reddy et al., 1991) and *B. bovis* (Calder et al., 1996) were reported the DNA encoding these genes or their transcription unit (ribosomal DNA ; rDNA)was amplified from genomic DNA. In Egypt, bovine babesiosis has been considered as most important and endemic parasitic disease affecting cattle, mainly *B. bigemina* and *B. bovis* (Nagati, 1947). The former is much common than the latter (Eýzzat, 1960). Diagnosis had been developed based on examination of stained blood smears or serological tests by many investigators (Sakla, 1975 ý, Nassar , 1992 , El- Ghaysh, 1993 and fadly 1996). The present study was undertaken to describe the use of Polymerase Chain Reaction (PCR) to specifically amplify a region of DNA encoding the SSr RNA genes of *B.bovis* and *B.bigemina* from genomic DNA. The specificity and sensitivity of the test were standardized on positive control cases and subsequently in carrier animals in attempt to compare it with stained blood smears.

MATERIALS AND METHODS

1) Collection of the samples:

a) Control samples :

Ten mls.of jugular blood were collected from two cattle (5 ml. per each) (in a private farm belonging to Giza governorate) in two evacuated tubes containing ethylene diamintetraacetic acid (EDTA) as anti coagulant. These two animals showed clinical symptoms of babesial infection and confirmed by Giemsa stained blood smears. These animals were considered as positive control. The blood of each was aliquoted in siliconized microcentrifuge tubes (one ml. per each) prior to DNA extraction. These tubes were centrifuged at 4.000 r.p.m. for 15 min.; the

plasma and buffy coat were discarded. The pellets of red blood corpuscles (Rbcs) were washed in Phosphate buffer saline (PBS) pH 7.4 by centrifugation at 14.000 r.p.m. for 15 min. and discard the supernatant. The tubes were labeled and frozen at - 70 C until use (Calder, 1994).

b) Test samples:

110 clinically normal Egyptian cows from Giza governorate of different ages were bled (5 ml. blood per each). These samples were processed as described above in control samples (Calder, 1994) Giemsa stained blood smears were prepared and examined from each animal.

2) Samples preparation:

Samples were processed as previously described (Calder, 1994 and Calder et al 1996). Briefly one frozen Rbcs. pellet of each control case was lysed by two rapid freeze-thaw cycles with centrifugation at 14.000 r.p.m. for 15 min. each cycle. The cell debris and/ or the parasite were washed twice with 1 ml. of Tris-EDTA buffer (TE) pH 8 (10 mM tris-HCL pH.8 and 1mM EDTA pH.8) DNA was extracted from the final pellets using guanidine thiocyanate (Fluka cat #50990) and diatamaceous earth as silica (Sigma cat# D-5384) according to (Boom et al 1990). The DNA pellets were eluted in 100 ul of TE buffer and transferred to clean labeled siliconized tubes, stored at - 20 C until used.

3) Synthetic oligonucleotides :

PCR primers and probes prepared for this study were listed (Table 1). All primers and oligonucleotide probes were synthesized on the sequences of *B.bovis* and *B. bigemina* (Mexico strain) in Applied Biosystems DNA Synthesizer at the Interdisciplonary Center for Biotechnology Research (ICBR) DNA Synthesis Core Facility at University of Florida and kindly provided by Drs: Roman R. Ganta and John B. Dame (Univ. of Florida USA).

4) Processing control samlpes for PCR:

Two sets of PCR primers were used: **A/B** (primer set **I**) which correspond to (SSr RNA)of *b. bigemina* (GenBank accession # X59604; Reddy et al 1991) and **C/D** (primer set **II**) correspond to (SSr RNA) of *B. bovis* (GenBank accession # L31922; Calder, 1994). In the initial reactions, final concentrations of each component in a 100-ul PCR mixture, a modified protocol from several sources (Saiki, 1988, Fahrimal et al 1992 and Calder, 1994) was 1 X PCR buffer, 2.5 unite of Taq-DNA polymerase, 200 uM (each) deoxynucleoside -triphosphateý (PCR Reagent Kit, Perkin - Elmer Cetus Part # N801-0055) and 2 uM of each species-specific PCR primer set (1uM per each). 20 ul of each of

processed control sample used as template (10 ul to the mixture containing primer set **I** and the other to the mixure containing primer set **II**. These templates were substituted by known DNA of *B.bigemina* and *B.bovis* ; Mexico strain and served as reference positive control for the PCR reactions. 50 ul of mineral oil (Sigma cat.# M-5904) was added to each tube to prevent the evaporation of the samples subjected to the high temperature. The parameters for thermocycling following an initial 10 min. at 93 C were modified from some protocols (Fahrimal et al. 1992 and Calder 1994): 1 min. at 93 C (denaturation), 1:30 min. at 49 C (primer annaeling) ,and 3 min. at 72 C (primer extension and polymerization) which was repeated 35 times; after the last cycle, the mixture was heated once at 72 C for 10 min. and then held at 4 C until use.The temperature program was run in an 110S thermacycler (Coy Corp., Grass Lake, MI, USA).

Ten microliters of each PCR product was analyzed in a duplicate manner by electrophoresis on a 1% agarose gel in buffer containing ethidium bromide (1ug per ml.) for 2 hours at 150 volts. Photographed under ultraviolet (UV) light. The gels were subsequently transferred onto 0.45 um. Quantum yield membrane (Promega Corp.), then UV light cross linked according to (Sambrook et al., 1989).

5) Probe preparation and blot hybridization :

Species specific oilgonucleotide probes **(E & F)** corresponding to *B.bigemina* and *B.bovis* respectively were prepared by its conjugation with alkaline phosphatase to each of these two probes separetly with the nonradioactive (NR) Light Smith I Kit (Promega Corp., Madison, Wis, USA) according to the manufacture's instructions (Cate et al., 1991) and susequently, the (NR) detection was done according to the protocol of used Kit.The bloted membrane was cut into two pieces corresponding to the duplicated gels. Add each probe to its membrane to give a final concentration of 500 femto mol. per ml. hybridization buffer (Calder et al., 1996), and this was hybridized at 35 C for 45 min . Autoradiograms were prepared by exposing the blot to X-OMAT film (Kodak, cat.#165-1678) for one hour at 37 C (Calder, 1994) .Unfortunately, one of the control samples was mixed infection. So one of the test samples confirmed to be single infection by PCR will substitute the mixed control one to make correct serial dilution. DNAs concentration of these single infection samples were determined spectrophotometrically in (Spectronic 601 Milton Roy., USA) according to (Sambrook et al., 1989). To determine the limits of detection for the PCR. The DNAs of single infection positive control for *b.bovis* and positive test sample for *B.bigemina* were serially diluted in TE buffer to yield sets of dilutions

per 10 ul.as follows; 10 ng (nanogram), 1 ng, 100 pg (picogram),10 pg,1 pg and 100 fg (femtogram). Ten microliters of each concentration from each species were subjected to PCR using the two species PCR primers. After cycling, the PCR products were electrophoresed, blotted and hybridized with its species specific probes as described in the control samples.

6) Processing test samples for PCR :

One frozen Rbcs pellet of each test samples were processed , DNA extraction, PCR set up, blotted and hybridized as described above.

RESULTS

Specificity of the PCR:

The specificity of *B. bigemina* PCR primer set **I** and *B.bovis* set **II** (Table 1) verified from the sequence of SSrRNA genes of Mexico strain of both species were tested with DNAs extracted from blood samples of two clinically infected Egyptian cows (1ml. per each) confirmed microscopically babesia positive. DNAs of *B.bigemina* and *B.bovis*,Mexico strain used as reference control (Fig. 1). The expected 175 base pair (bP) and 275 bp fragments for both species respectively were visualized in agarose gel. Both bands generated from the reference control DNAs and one Egyptian control sample and only the 275 bp fragment was visualized in the other one. This indicated that, one Egyptian control sample had mixed infection and the other had only *B.bovis* infection. (Fig. 1.A). The specificity was confirmed by Southern nonradioactive hybridization with the respective species specific probes (**E & F** Table 1). The probes react specifically with PCR products. Detectable signals at 175 bp for *B.bigemina* (Fig 1.B) and 275 bp for *B.bovis* (Fig 1.C) were generated . Both signals were observed in the reference control and only one Egyptian control sample . On the other hand, one signal was detected for *B.bovis* in the other one

Sensitivity of the PCR:

The reliability of detection at various dilutions of DNAs from single infection control sample with *B.bovis* and one test sample infected with *B.bigemina* (confirmed by PCR) was evaluated . The DNAs from the two species were serially diluted separetly from 10 ng to 100 fg. per 10 ul.extracted DNA. The density of the amplified fragments were decreased gradually with lowering the dilution of DNAs subjected to PCR .

In *B.bigemina* ,the expected 175 bp fragments were detected. strong signal was observed at 10 ng. Very faint fragment was visualized at 100 fg. (Fig. 2,B). The density was decreased gradually. Southern hybridization with specific probe (**E** . Table 1) gave the same degree of signal density (Fig 2 ,C) corresponding to the serial dilutions and the density of PCR products. At lowest detection limit (100 fg.) in 10 ul. extracted DNA implies the lower number of parasites could be detected in one ml.blood. Ten microliters of eluted DNA contain about 1.5 parasite (66 fg per parasite) with totally 15 parasites per one ml. blood.

In *B.bovis*, The expected 275 bp fragments were visualized. strong band was observed at 10 ng. and the density was decrease gradually.Very faint fragment obtained at 1 pg. (Fig2,A). Southern hybridization with respective probe (**F**.Table1) gave the same degree of signal density (Fig.2,C) corresponding to the serial dilutions and the density of amplified fragments. The lowest detection limit (1 pg) in 10 ul.extracted DNA subjected to PCR implies the lower number of parasites could be detected . The DNA extracted from one ml. blood was eluted in 100 ul.TE buffer, 10 ul of them contain about 20 parasites (50 fg per parasite) and subsequently 200 parasites per one ml.blood.

Table 1 PCR primer set s and oilgonucleotide probes used.

Name *	Sequence **	Position(nt) #	Species	Size(bp) ##
A	5'TGTCCTCGTTTGCTTCTTAGAGGGACTCCT3'	1488-1517		
			B.bigemina	175
B	5'CCGACACGATGCACACTAAACATTACCCAA3'	1635-1664		
C	5'TTGGCATGGGGGCGACCTTCACCCTCGCCC 3'	450-479		
			B.bovis	275
D	5'CCAAAGTCAACCAACGGTACGACAGGGTCA3'	692-721		
E	5'GCATCCATCGAGTTCGTCCTGTCC 3'	1603-1626	*B.bigemina*	
F	5'GCAGGTTTCGCCTGTATAATTGAGC 3'	629-653	*B.bovis*	

* A/B are primer set I , C/D are primer set II
A and C are forward PCR primer , B and D are reverse PCR primer.
The arrangment of deoxynucleotide- triphosphate; **C -- Cytosine , **A**--Adenine **T**--Thiamine and **G**--Guanine . **5'** and **3'** : number of carbonatom in the nucleotide at which the sugar phosphate backbone attached to form deoxynucleotide-triphosphate.
Positions for the *B.bigemina* SSrRNA gene(GenBank accession # x59604) and *B.bovis* SSrRNA gene (GenBank accession # L31922)
Expected size is from the begining of forward to the end of reverse primer
E : Single oligonucleotide for *B.bigemina* and **ýF:** for *B.bovis*

Detection of carrier Cattle by PCR and blood film examination:

The PCR primer sets (**I & II**) and probes (**E & F**) were used to detect DNAs of parasite populations in the clinically normal Egyptian cows. The expected 175 bp (Fig 3,A) and 275 bp (Fig 3,B) fragments were visualized in agarose gel electrophoresis corresponding to *B.bigemina* and *B.bovis* respectively. The sensitivity of *B. bigemina* probe was very high, it detected a very faint non-visualized PCR fragments (Fig 3. C). The PCR products of *B.bovis* showed non specific bands with the expected one (Fig 3, B). Southern blot and hybridization with corresponding (NR) probe, revealed strong defined signals.The non specific bands couldn't give any signal following the autoradiograms (Fig 3, D). 33 out of 110 (30 %) blood samples showed the expected band of *B. bovis* and 45 out of 110 (40.9%) observed the specific band of *B. bigemina*. On the other hand, 5 out of 110 (4.5 %) showed mixed infection. Whereas, 2 out of 110 (1.8 %) cows were positive by Giemsa-stained blood smears. These two cases, the parasite couldn't be differntiated microscopically. By PCR the parasite was identified as *B. bigemina*.

DISCUSSION

Postacute, carrier cattle infected with *Babesia* species are difficulty detected because of the low number of parasites that occur in peripheral blood by traditional methods as microscopic examination (Sakla, 1975, Gattas, 1990 and Fadly 1996) or serological tests (Chafick,198, Nassar, 1992 and Fadly, 1996). However, diagnosis of this animal status was important for evaluating the efficacies of vaccines and in transmission and epidemiological studies. The advent of molecular biology opened a new approach to the diagnosis and species identification of *Babesia* species by permitting detection of genetic blueprint of the causal agent. The availability of nucleotide sequences enhanced the development of PCR and probe hybridization for rapid detection and differntiation of *Babesia species*. (Reddy et al., 1991, Fahrimal et al., 1992, Figueroa et al., 1993 and Calder 1994).

We described a sensitive method to detect *B.bigemina* and *B.bovis* carrier Egyptian cattle by PCR amplification using primers and probes verified from the sequence of Mexico strain of both species (Reddy et al., 1991 and Calder 1994) for detection of the transcription unit of the DNA encoding the SSrRNA gene from genomic DNA of each species separetly.

The current tests in our study that used PCR amplification, Southern blotting and hybridization fullfill most of the criteria of sufficent diagnosis. The method of DNA extractionis quite simple and fast where extraction of a single sample takes less than 10 min.This agrees with (Calder 1994) and disagrees with (Sambrook et al., 1989) who used sodium dodecyl sulfate (SDS) and Proteinase K. for over night extraction. Regarding the specificity of PCR; we amplified 175 bp and 275 bp fragments corresponding to *B.bigemia* and *B.bovis* respectively from both clinically infected Egyptian cows confirmed microscopically babesia positive and the reference control by using of primer sets **I & II** (Table 1). These two bands were exactly generated from Mexico strain of the same species using the same primer sets (Calder, 1994) with some minor modifications in annaeling temperature of PCR and hybridization. The successful amplification and hybridization of the Egyptian strain using these primers may atributed to the great similarity or sequence homology in the transcription unite of DNA encoden the SSrRNA genes in various strains. This opinion was supported by (Calder, 1994) who found the degree of sequence similarity of DNA encoden SSrRNA gene between different strains of *B.bigemina* and that of *B.bovis* were extended from 88% - 97%. On the other hand , we used these primer sets based on the sequence of SSrRNA gene to amplify its transcription unit of DNA in *B.bigemina* and *B.bovis* were identified as potential targets for developing a diagnostic test for babesiosis . This was identical to that reported by (Waters and McCutchan, 1990 and Calder, 1994) who stated that 90 % - 95% of total RNA in the cell is ribosomal RNA and subsequently their transcription unit of DNA. Moreover, rRNAabundance is 50% time more than the chromosomal DNA in the cell. The using of PCR was significantly improved the sensitivities for detecting *B.bigemina* and *B.bovis* DNAs which implied the number of parasites in bovine blood. In our study, the lowest detection limit in *B.bigemina* was 100 femtogram per 1/10 DNA extracted from one ml.blood. This was implied about 15 parasite per one ml.blood on the calculation of (Buening et al., 1990) who mentioned that 10 picogram equivalent of 15 parasite (66 fg.per parasite). On the other hand, the lowest detection limit in *B.bovis* was one picogram per 1/10 DNA extracted from one ml.blood. This was reflected 200 parasite per one ml.blood based on the calculation of (Fahrimal et al., 1992) who described that 2000 parasite containing 200 picogram (50 fg. per parasite). This degree of sensitivity is higher in *B.bigemina* than that obtained by (Calder 1994 - 100 parasite per ml.blood) and lower in *B. bovis* than that recorded by the same author- 100 parasite per ml.blood .

Whereas, this sensitivity was higher than that observed in the initial using of SSrRNA as a tool for diagnosis (Reddy and Dame, 1992) who detected 6000parasite per ml.blood for *B.bovis* and 100 parasite per 20 ul.of blood for *B.bigemina*.

The discripancy in the sensitivity may be ascribed to standerdizing and optimizing the condition of PCR and hybridization temperatures. In our opinion, the PCR primers were not perfectly annaeled to the DNA molecules subjected to PCR as the original sequence. Failure of annaeling in some DNA molecules lead to lowering the sensitivity. On the other hand , lowering the annaeling temperature in PCR condition incerase the probability of primer annaeling (Saiki et al., 1988) and subsequently increase the sensitivity. In our study the annaeling temperature was 49 C instead of 60 C used by (Calder, 1994). In fact , we lowering the recommended annaeling temperature from our belives that these primers not verified from our SSrRNA gene corresponding to bovine babesiosis in Egypt . Moreover,the level of 100 or 200 parasite of *B.bigemina* in one ml.blood was completly micriscopically undetectable (Mahoney, 1969).

Concerning the applicability of PCR for diagnosis; successful amplification of 40.9% of blood samples for *B.bigemina* and 30% for *B.bovis* were detected. This reflected that the former was common than the latter in Egypt. The achieved results coincide with those recorded by (Abd El-Gawad, 1993) in Beni- Suef governorate. Similarly, 4.5 % of samples subjected to PCR showed mixed infection, a condition which may be difficult to perform by direct examination or serodiagnosis due to interspecies cross reaction. This coincides with that reported by (Todorovic, 1975 and Morzaria et al., 1992).

In general, all the PCR amplified fragments were confirmed by Southern blot hybridization with species specific non radioactive probes. The density of the signals simulated the amplified fragments;a similar results were obtained by (Calder,1994).

The microscopical examination of Giemsa-stained blood smears from all tested samples resulting in 1.8 %. In contrary, high incidence was recorded microscopically than in our study by many authors in Egypt. (Sakla 1975, Gattas,1990, Abdel-Gawad, 1993 and Fadly, 1996). In conclusion, we developed a PCR-based method for the direct detection of *B.bigemina* and *B.bovis* carrier cattle that is superior to existing methods as microscopical examination and serological techniques. The method is highly sensitive and broadly applicable to strains of the parasite from diverse geographic regions. The sensitivity of this method will facilitate analysis of vaccins and their ability to induce or prevent the

carrier state. Additionally, this method may be used for testing the efficacies of drugs against the parasite and in studies on the transmission and epidemiology of the disease.

ACKNOWLEDGEMENTS

I would like to thank Drs: Roman Reddy and John B. Dame, Department of Pathobiology, College of Vet. Med. Univ. of Florida, Gainesville, Fl 32610 for their advice and help in getting this project completed by their kindly providing the PCR primers, probes and the non-radioactive kit. I thank Dr: Jennifer Ann Marie Univ. of Florida for her providing a boody of literatures and her Ph D disseration.

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Fig. 1: **(A)** Ethidium bromide stained gel of PCR amplified control samples using primer set I &II for *B.bigemina* and *B.bovis* DNA. The PCR products were run on left side for *B'.bigemina* and on right side for *B. bovis* Lane (1) reference positive control (Mexico strain) Lanes (2 & 3) Egyptian positive control and (M); size marker (174/HaeIII, Hind III Stratagene # 201102) and its bands are 1078, 872, 603, 281, 194 and 125 bp indicated by dashes from top to bottom respectively. The black arrows showed the *Babesia species* PCR amplified fragments.**(B)** Southern blot hybridization of panel **(A)** with **E** species specific probe. **(C)** Southern blot hybridization of panel **(A)** with the **F** species specific probe. Black arrows showed the signals of *Babesia species.*

Fig. 2: Ethidium bromide stained gels of PCR amplified serial dilutions of *B.bovis* and *B. bigemina* DNAs **(A & B)**. Southern blot hybridization with nonradioactive probes visualized on X-ray film **(A)** for *B.bovis* and **(B)** for *B.bigemina.*(M); 123 bp marker in base pair (life Technologies. Inc., Bethesda, MD) and its bans are 123, 246 and 369 bp from bottom to top respectively. Lanes (1) 10 ng., (2) 1 ng., (3) 100 pg., (4) 10 pg., (5) 1 pg., and (6) 100 fg. (each per 10ul DNA). Balck arrows showed *Babesia species*amplified fragments. Autoradiograms of panel **A** and **B** with species specific probe **F** for *B.bovis* and **E** for *B.bigemina.* The density of the signal represent the amplified fragments, black arrows showed the signal of *Babesia species.* **C,** for *B, bovis* and **D,** for *B. bigemina.*

Fig: 3: Ethidium bromide stained gels of PCR amplification DNA extracted from blood of some carrier animals using primer set **I** for *B,bigemina* **(A)** and primer set **II** for *B.bovis* **(B)**. (M) is size marker (123 bp marker). **(C)** Southern blot hybridization of panel **(A)** with **E** probe for *B.bigemina* and **(D)** southern blothybridization of panel **(B)** with **F** probe for *B.bovis* .Black arroes showed the PCR fragments and signals . Lanes 1 - 9 showed mixed infection, Lane 13 showed *B.bovis* only and Lanes 10-12 showed negative samples.

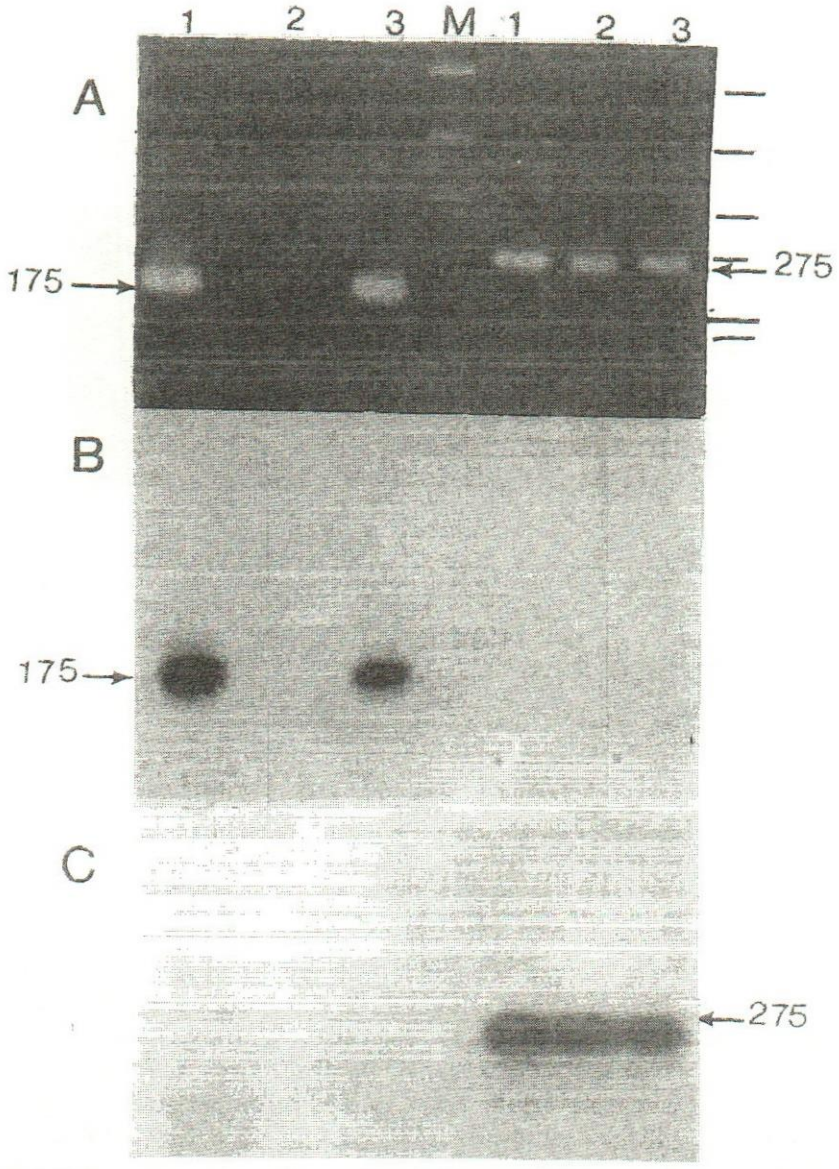

Fig :1 (A) Ethidium bromide stained gel of PCR amplified control samples using primer set I &II for *B.bigemina* and *B.bovis* DNA. The PCR products were run on left side for *B.bigemina* and on right side for *B. bovis* Lane(1) reference positive control (Mexico strain) Lanes(2 & 3) Egyptian positive control and (M); size marker (174/HaeIII, Hind III Stratagene # 201102) and its bands are 1078 , 872 , 603 , 281 , 194 and 125 bp indicated by dashes from top to bottom respectively. The black arrows showed the *Babesia species* PCR amplified fragments.(B) Southern blot hybridization of panel (A) with E species specific probe. (C) Southern blot hybridization of panel (A) with the F species specific probe. Black arrows showed the signals of *Babesia species.*

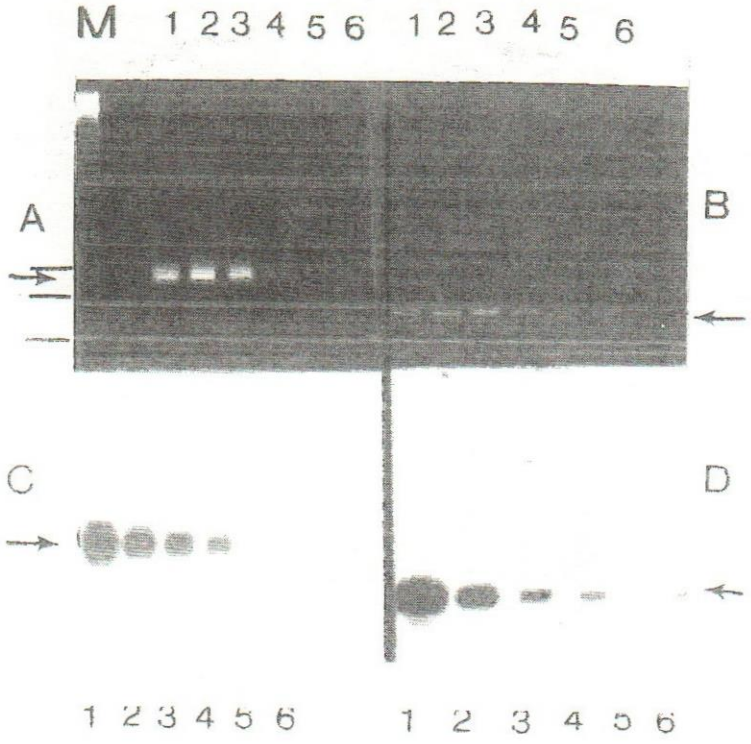

Fig : 2 Ethidium bromide stained gels of PCR amplified serial dilutions of *B.bovis* and *B. bigemina* DNAs (**A & B**). Southern blot hybridization with nonradioactive probes visualized on X-ray film . **(A)** for *B.bovis* and **(B)** for *B.bigemina.*(M); 123 bp marker in base pair (life Technologies. Inc., Bethesda, MD) and its bans are 123 , 246 and 369 bp from bottom to top respectively.Lanes (1) 10 ng. , (2) 1 ng., (3) 100 pg., (4) 10 pg., (5) 1 pg., and (6) 100 fg. (each per 10ul DNA). Balck arrows showed *Babesia species*amplified fragments. Autoradiograms of panel **A** and **B** with species specific probe **F** for *B.bovis* and **E** for *B.bigemina* . The density of the signal represent the amplified fragments, black arrows showed the signal of *Babesia species* . **C** , for *B. bovis* and **D**, for *B. bigemina.*

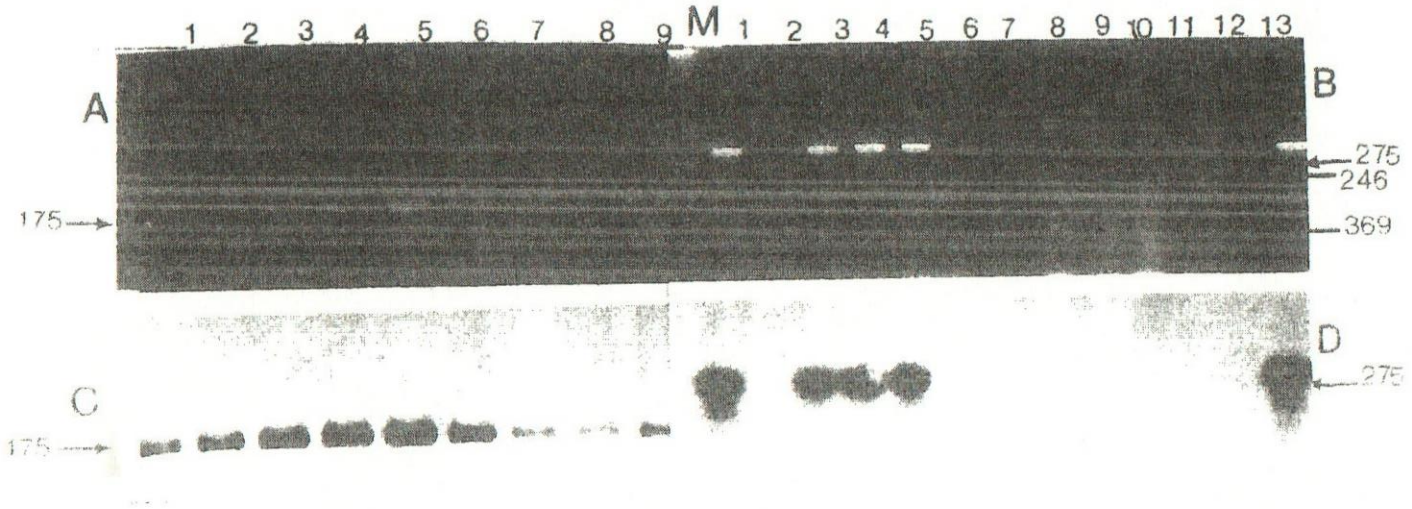

Fig : 3 Ethidium bromide stained gels of PCR amplification DNA extracted from blood of some carrier animals using primer set I for *B,bigemina* (A) and primer set II for *B.bovis* (B). (M) is size marker (123 bp marker) . (C) Southern blot hybridization of panel (A) with E probe for *B.bigemina* and (D) southern blot hybridization of panel (B) with F probe for *B.bovis* .Black arroes showed the PCR fragments and signals . Lanes 1 - 9 showed mixed infection, Lane 13 showed *B.bovis* only and Lanes 10-12 showed negative samples.