Assiut Vet. Med. J. Vol. 42 No. 83, October 1999

Parasitology Department, Biotechnology Lab.
Animal Health Research Institute, Dokki, Giza.
Agricultural Research Center, Cairo, Egypt.

DIAGNOSIS OF BOTH *BABESIA BOVIS* AND *BABESIA BIGEMINA* INFECTIONS AMONG CARRIER CATTLE BY USING EXTRA-CHROMOSOMAL DNA-BASED POLYMERASE CHAIN REACTION TEST IN EGYPT.

(With 3 Figures)

By
G.H. SALEM

(Received at 29/9/1999)

تشخيص العدوى بطفيل البابيزيا بوفس والبابيزيا بايجيمينا فى الأبقار الحاملة للمرض باستخدام الحمض النووى خارج النواة المعتمد على أختبار تفاعل البلمرة المتسلسل فى مصر

جمال حلمى سالم

الأبقار الحاملة للعدوى بطفيل البابيزيا بوفس والبابيزيا بايجيمينا من الصعب تشخيصها نظرا لقلة عدد الطفيل فى دم الشعيرات الدموية ومن ذلك تشخيص وأكتشاف هذا العدد القليل من الطفيل له أهمية كبيرة فى تقييم التحصينات ودراسة وبائية المرض فى مصر . أستخدمت هذه الدراسة تفاعل البلمرة المتسلسل لإظهار جزء من جين الأبوسيتوكروم بى الموجود فى جميع طفيليات الدم وبالأخص نوعى البابيزيا فى تفاعل واحد وذلك بأستخدام طقم بوادىء عام مصمم من مناطق متشابهة لتتابعات هذا الجين فى نوعي البابيزيا (عترة المكسيك) وقد تم أختبار أمكانية هذا الطقم فى أكتشاف العدوى بالعترات المصرية فى ضوابط مصابة بالطفيل تم التأكد من إصابتها ميكروسكوبياً وقد تم أكثار نفس الجزء من الجين لجنس البابيزيا فى مصر فى هذه الضوابط بنفس الحجم بالمقارنة بالعترات المرجعية وقد تم التفرقة بين نوعى الطفيل بأستخدام مجس متخصص لكل طفيل على حدى . وفى محاولة لمعرفة مدى حساسية هذا الإختبار وجد أنه بإستخدام تفاعل البلمرة المتسلسل بالأشتراك مع التهجين بالمجسات المتخصصة الغير مشعة أمكن إكتشاف كمية من الحمض النووى الديوكسي ريبوزى (DNA) مقابلة لطفيل واحد من كل نوع على حدى فى ١ مللى دم . وبأستخدام هذا التفاعل فى الحيوانات الحاملة للمرض وجد أن ٣٠,١ % من العينات أيجابية لطفيل البابيزيا بوفس فقط , ٤٠,٢ % لطفيل البابيزيا بايجيمينا منفرداً و ٦,٠٢ % كانت مصابة بالطفيلين معاً . وقد وجد بالفحص الميكروسكوبى ان ٢,٤ % من جميع العينات إيجابية للبابيزيا والتى أمكن التعرف عليها بأستخدام أختبار تفاعل البلمرة المتسلسل . وقد أكدت النتائج أن هذا الأختبار له حساسية عالية لإكتشاف أقل عدد من الطفيل فى دم الحيوانات الحاملة للمرض

فى مصر وكذلك التفرقة بين الأنواع المختلفة للطفيل . وقد أشارت الدراسة أن صفات جين الأبوسيتوكروم بى لها أهمية كبيرة جداً بالأعتماد على أختبار تفاعل البلمرة المتسلسل فى أكتشاف بعض طفيليات الدم الأخرى فى مصر .

SUMMARY

Carrier cattle infected with *Babesia bovis* and/or *Babesia bigemina* are difficult to detect because of the low numbers of parasites in peripheral blood. Diagnosis of these carrier status is important for evaluating the efficacies of vaccines and in the epidemiological studies. The present study used the polymerase chain reaction (PCR) to amplify a portion of 644 base pair (bp) of the apocytochrome b gene from both *Babesia* spp. in one PCR reaction using a common PCR primer pair set conserved in both *Babesia* spp. and tested the ability of this method to detect the Egyptian strains. The same amplified band was generated and identified by Southern blot hybridization with non-radioactive species specific probes on the Egyptian control samples. The sensitivity of the extra chromosomal DNA-based PCR test was 50 femtogram of DNA / 100 µl extracted genomic DNA of each parasite independently from one ml blood. This was equivalent to one parasite from each species. The PCR assay followed by Southern blot hybridization identified that 30.1 % of the carrier cattle harboured *B. bovis* only and 40.2 % had only *B. bigemina* as well as 6.02 % had mixed infections with overall rate of 78.3%. Moreover, microscopic examination of blood smears of the same carrier cattle showed the parasites only in 2.4 % of the total samples. This PCR method provides a useful diagnostic tool for detecting carrier cattle infected with *B. bovis* and/or *B. bigemina* and the sensitivity is significantly improved over that of current methods. The present investigation also suggest that characteristics of the apocytochrome b gene may make this available target DNA for PCR-based detection of other hemoparasites in Egypt.

Key words: *Babesia bovis, Bigemina, Extra-chromosomal DNA.*

INTRODUCTION

Babesiosis is caused by tick-borne protozoan hemoparasites of genus *Babesia* (Wright and Riddles, 1986). *Babesia bovis* and *Babesia bigemina* are responsible for bovine babesiosis . The disease is endemic and continues to be a significant deterrant to livestock production in developing countries with tropical and subtropical climates (Kuttler,

1988). After recovery, a long-lasting carrier status occurs, which may difficult to be detected microscopically (Mahoney, 1969 and Young, 1988). Traditional methods for detection of the parasites in these animals depend on indirect serological tests (Bose et al., 1990). These tests exhibited lack sensitivity and specificity (Morzaria et al., 1992; Calder et al., 1996 and Salem et al., 1999).

Improved methods to detect the parasites will aid epidemiological studies of babesiosis and will be useful for assessing efficacy of drug and vaccine treatment regimens (Fahrimal et al., 1992). Recently, molecular biology methods are creating possibilities to the development of deoxyribonucleic acid (DNA) probes from repetitive genomic DNA of *B. bigemina* and *B. bovis* (Buening et al., 1990 and Jasmer et al., 1990). However, these probes increase the sensitivity of detection but unlikely to be adequate for the detection of carrier animals (Figueroa et al, 1998).

The advent of polymerase chain reaction (PCR) offers an alternative approach for development of sensitive diagnostic assays for *B. bovis* and *B. bigemina* (Innis et al., 1990; Fahrimal et al., 1992; and Salem et al., 1999). In addition to the nuclear DNA, parasites have an unusual mitochondrial DNA called extra-chromosomal DNA with identical sequences and the genes are represented in many copies (Lodish et al., 1995). The mitochondrial genetic systems of the Apicomplexa examined so far are proving to be fascinating consisting of a conserved 6 – 7 kilobase DNA element (Jasmer et al., 1990; Megson et al., 1991 and Salem et al., 1999). The apocytochrome b gene sequence in the extra-chromosomal DNA may be a useful target for PCR-based detection of many hemoparasites (Joseph et al., 1989; Megson et al., 1991; Fahrimal et al., 1992 and Salem et al., 1999).

In Egypt, babesiosis is an endemic parasitic disease of cattle and buffaloes caused by *B. bovis* and *B. bigemina* (Nagati, 1947). Currently, detection of the infections were mostly based upon examination of Giemsa stained blood smears or serological tests (El-Allawy, 1973; Sakla, 1975; Chafick, 1987; Fadly, 1996 and Mohran, 1998). Moreover, Salem (1998) described the use of PCR for detection of both parasites infected cattle using the Small Subunits ribosomal ribonucleic acid (SSrRNA) genes as targets.

The present work was conducted to use the PCR for sensitive and specific amplification of an extra chromosomal DNA segment from *B. bovis* and *B. bigemina* in the same PCR reaction using a common genus specific primer pair. The specificity and sensitivity were estimated on clinically infected cattle and serial dilutions of genomic DNA of

parasites. Finally, PCR was compared with stained blood smears by using a panel of blood samples from carrier cattle.

MATERIALS and METHODS

Source of blood samples:

Two groups of cattle from a private farm of native cows at Beni Suef Governorate were used in the present study. The first group of three cows had clinical symptoms of babesiosis and were proven to be infected microscopically. These animals considered as positive control. The second group of eighty three clinically normal cows. Giemsa stained blood smears from each animal from this group prepared and examined microscopically.

Three ml of blood were bled from each animal of the two groups in evacuated tubes containing ethylene diamintetraacetic acid (EDTA) as anticoagulant. The blood of each animal was aliquoted in microcentrifuge tubes (one ml/each). Prior to DNA extraction, centrifugation and discarding the plasma and the buffy coat were done for all tubes. The red blood corpuscles (RBCs) pellet equivalent to one ml blood of each tube was washed in phosphate buffered saline (PBS) pH 7.4 by centrifugation, removal the supernatant and frozen at - 70°C until use (Calder, 1994).

DNA isolation:

One frozen RBCs pellet of each control samples as well as of the clinically normal one were lysed and washed according to Calder et al. (1996). From the final pellets, extraction of DNA was done using diatamaceous earth or silica (Sigma Cat # D-5384) and guanidine thiocyanate (Fluka Cat # 50990) following Boom et al. (1990). The DNA pellets were resuspended in 100 µl TE buffer (Tris-EDTA) pH 8.0 (10 mM tris HCL pH 8.0 and 1 mM EDTA pH 8.0) and transferred to siliconized tubes, labeled and stored at - 20°C until used.

Synthesis of PCR primers and oligonucleotide probes:

The common PCR primes and oligonucleotide probes designed for this study were obtained from the sequence of a portion of apotcytochrome b gene of *B. bovis* (Jasmer et al., 1990, GenBank # AF053002) and that of *B. bigemina* (Salem et al., 1999, GenBank # AF109354). This primer pair set was designed from the regions conserved in both *Babesia* spp. (Mexico strain) to amplify a fragment of 644 base pair (bp) from both *Babesia* spp. A forward primer 5′TGGAAC/TTTAGGGTTTATA/CG 3′ (nucleotide position 1 to 19)

and a reverse primer 5'GGT/AATTACTCCATAAGTTA3' (nucleotide positions 626 to 644). Species specific oligonucleotide probes were verified to identify species specific PCR products as 5'GAGTGCTTGCTGAAGTCAATATGG 3' (from nucleotide 113– 136) corresponding for *B.bovis* and 5'GAGTATTAACTGAGGTTAATATGG3' (from nucleotide 113–136) specific for *B. bigemina*. The common PCR primers and species specific aligonucleotide probes were synthesized in Applied Biosystems DNA Synthesizer at the Interdiscriplonary Center for Biotechnology Research (ICBR) DNA Synthesis Core Facility at University of Florida and kindly supplied by Drs.: Roman R. Ganta and John B. Dame (University of Florida, USA).

PCR amplification of the control samples:

Polymerase chain reactions were achieved in a final reaction volume of 100 µl with a final concentration of each component as described by many authors (Saiki, 1989; Innis et al., 1990 and Fahrimal et al., 1992) was 2.5 unit of Taq DNA polymerase, 200 µM (each) deoxynucleoside-triphosphate (PCR reagent kit, Perkin-Elmer Cetus Part # N801-0055) and 1 µM of the common forward primer and 1 µM of the common reverse one. Ten microliters of each processed Egyptian control sample used as a template DNA. A known DNAs of *B. bovis* and *B. bigemina* (Mexico strains) were used as reference positive control instead of the Egyptian control samples. Moreover, for the PCR reaction kit, a control template of the kit with its specific primers to amplify a band of 720 bp was used as described before. To prevent the evaporation of the samples undergo the high temperature during amplification, 50 µl mineral oil (Sigma Cat # M-5904) was added to each of the three Egyptian control samples, two references control and the control of PCR kit. The reactions were performed in an automatic 110S DNA thermacycler (Coy Corp, Grass Lake, MI, USA) for 35 cycles. The parameters for thermocycling following an initial 10 min. at 93°C was as described by Salem et al. (1999).

Detection and characterization of PCR products:

Ten microliters of each amplified products of all the control samples were separated by electrophoresis in duplicate on a 1 % agarose-gel containing ethidium bromide (1 µg/ml) for an hour at 180 Volts and then photographed under ultra violet (UV) light. The size of the PCR bands were calibrated on semilogarethmic paper. The gels were transferred by Southern blotting onto Quantum yield nylon membrane 0.45 µm (Promega Corp) and cross-linked with UV light according to Sambrook et al. (1989).

The blotted membrane was cutted into two parts represent the duplicated gel. The oligonucleotide probes specific for *B. bovis* and *B. bigemina* were prepared and conjugated with alkaline phosphatase separately using nonradioactive chemiluminescence (NR) Light Smith I Kit (Promega Corp Madison, Wis, USA) according to Cate et al. (1991). Each membrane containing the PCR products was hybridized at 35°C for 45 min. with corresponding oligonucleotide probe (Calder et al., 1996). The membranes were exposed to X-OMAT film (Kodak Cat # 165-1678) for three hours at 37°C in order to enhance the detection of the invisible PCR bands and to evaluate the lowest detection limit of PCR (Sambrook et al., 1989 and Calder, 1994).

Sensitivity of the PCR method on control samples:

Fortunately, each of the Egyptian control samples harboured a single infection. The concentration of DNA from purified two samples with different single species of parasite were evaluated by spectrophotometer (Spectronic 601 Milton Roy, USA) according to Sambrook et al. (1989). To estimate the detection limits of the PCR, serial dilutions of these genomic DNA were done in Tris-EDTA (TE) buffer to give sets of dilutions per 10 µl as described, 100 pg (Picogram), 50 pg, 10 pg, 5 pg, 1 pg, 100 fg (Femtogram), 50 fg, 10 fg, 5 fg and 1 fg. Ten microliters of each dilution corresponding to each species were amplified using the *Babesia* specific PCR primers, analyzed through gel electrophoresis, blotted, cross-linked and hybridized to its corresponding species oligonucleatide probes as mentioned above.

Detection of carrier cattle:

PCR tests for diagnosis were performed on 10 µl of DNA from each of clinically normal cattle using the same primer pair, gel electrophoresis in duplicate manner, blotted and hybridized each member as described in the control samples.

RESULTS

PCR amplification of *Babesia* spp. from control samples:

In the initial amplifications with the common PCR primer set of genus *Babesia* to genomic DNAs of the Egyptian and reference positive control (Mexico strain) samples, the expected 644 base pair (bp) band was detected in agarose gel in all the control groups and a 720 bp fragment of the control PCR kit was observed. The ethidium bromide-stained agarose gel showed some non-specific amplification of the Egyptian control samples beside to the expected 644 bp band (Fig. 1.A).

Species specificity of the probes:

Southern blot nonradioactive hybridization of the amplified DNAs from control samples gave detectable signals corresponding to species specific oligonucleotide probes. Each of the Egyptian control samples harboured a single infection. The specific probe for *B. bovis* identified one of the reference control samples have *B. bovis* as well as two of the Egyptian control samples (Fig. 1.B). Likewise, the specific probe for *B. bigemina* gave strong signals with the reference control harboured *B. bigemina* DNA and the other one Egyptian control sample (Fig. 1.C). There were differences in the intensities of the probes signals for the amplified products among the control samples. The control PCR kit band didn't react with both oligonucleotide probes.

Sensitivity of the PCR method:

To determine the detection limit of the PCR, serial dilutions of genomic DNA of *B. bovis* and *B. bigemina* of two Egyptian control samples were used. The expected amplified 644 bp fragments were detected by electophoresis. The intensities of the amplified bands were gradually decrease with increasing the dilution of DNAs undergo PCR method. In *B. bovis*, the PCR bands were very clear from 100 picogram to 100 femtogram and the other amplified fragments were ranged from very faint to invisible ones (Fig. 2.A). The lowest detection limit of nonradioactive Southern blot hybridization with the specific probe was 5 fg/10 µl of genomic DNA subjected to PCR, equivalent to 50 fg in 100 µl of genomic DNA that extracted from one ml blood. This was representing one parasite per one ml blood as each parasite had 50 fg DNA. There were markedly decrease in the signal intensities following that of the amplified bands (Fig. 2.B). Although some PCR bands were not clear by gel electrophoresis, they could be detected by Southern blot hybridization with prolonged exposure of the membrane to X-ray film. Moreover, in *B. bigemina*, the amplified products were ranged from very clear to faint bands from 100 pg to 10 fg and some bands were invisible (Fig. 2.C). Southern blot hybridization with specific probe for *B. bigemina* was 5 fg / 10 µl of genomic DNA undergo the PCR which equivalent to 50 fg in 100 µl of DNA extracted from one ml blood. This was corresponding to about one parasite in one ml blood as every 66 fg of DNA represent one parasite of *B. bigemina*. The signals of the hybridization followed the intensities of the amplified bands (Fig. 2.D).

Detection of carrier cattle:

The common PCR primer set of genus *Babesia* was used and performed on DNA extracted from a panel of blood samples taken from

clinically normal cattle. The amplified 644 bp fragments of *Babesia* spp. sometimes were generated clearly by gel electrophoresis (Fig. 3.A) but didn't reflect the proper rate of infection due to the probability of very low level of parasitamia in these carrier status which couldn't be visualized in the electrophoresis . Southern blot hybridization with specific probe for *B. bovis* with prolonged exposure of the membrane to X-ray film showed only 25 out of 83 (30.1 %) samples developed strong signals at the expected band (Fig. 3.B). On the other hand, the monoradioactive probe of *B. bigemina* gave signals at the expected bands by Southern blot hybridization with prolonged exposure of the membrane to X-ray film in 35 out of 83 (42.2%) samples. Whereas 5 out of 83 (6.02%) blood samples had mixed infections as they developed signals with both oligonucleotide probes (Fig. 3.C). The overall rate of *Babesial* infection was 65 out of 83 (78.3%) blood samples. However, only 2 of 83 (2.4%) blood smears showed the parasite microscopically. The parasite was identified as *B. bovis* in both samples by PCR.

DISCUSSION

The present study, developed a PCR assay for improved detection of *B. bovis* and/or *B. bigemina* in Egyptian carrier cattle by amplification and detection of an apocytochrome b DNA sequence belong to extrachromosomal DNA from both parasites in the same PCR reaction. A common set of PCR primers of genus *Babesia* was designed (Salem et al., 1999) from matching the sequence of this gene in *B. bovis* and *B. bigemina* . Mexico strain (Jasmer et al., 1990 and Salem et al., 1999 respectively) to allow amplification of a 644 base pair extrachromosomal DNA segment.

With this primer set, successful amplification of a 644 bp fragment from the genomic DNA of all the three clinically infected Egyptian cows. These amplified bands visualized in gel electrophoresis were as that fragments generated from Mexico strain of *B. bovis* and *B. bigemina* using the same primer set under the same condition of amplification. Moreover, the specificity of these fragments was confirmed by Southern blot hybridization with species specific probes which selectively identify the amplified products from both *Babesia* spp. These proven amplification and successful hybridization of the Egyptian strains may be ascribed to evidence of a similar sequences exist in the extrachromosomal DNA of *Babesia* spp. from different geographical regions. Matching to the sequence of *B. bovis* and *B. bigemina* to design this common PCR primer set from the regions

conserved in this gene of both *Babesia* spp. and the presence of relative abundance of sequence similarity between them may enhancing the successful amplification and proper hybridization to the Egyptian strains. This opinion was coincided with Jasmer et al. (1990); Fahrimal et al. (1992) and Salem et al. (1999), the latter found a degree of similarity more than 80% between the sequence of this gene in both parasites and the similarity between the sequence of these two parasites and *Theileria annulata* was more than 60%.

The present investigation showed differences in the signals intensities of the *B. bovis* probe for the amplified products among control samples (Fig. 1.B) which may represent uneven transfer of DNA to the nylon membrane as well as improper distribution of this probe on the blotted membrane, this finding came in close to Fahrimal et al. (1992).

Regarding the sensitivity of PCR based test described here for detection of the lowest limit of genomic DNA of each parasite. An approximately of 50 fg / 100 µl DNA extracted from one ml blood harboured *B. bovis* (5 fg / 10 µl DNA) which represented one parasite of *B. bovis* in one ml blood. On the other hand, 50 fg / 100 µl extracted DNA of *B. bigemina* which corresponding with one parasite in one ml blood. These were based on the calculation of Fahrimal et al. (1992) as 100 picogram of B. bovis DNA represented 2000 parasites (50 fg/parasite) as well as Buening et al. (1990) who estimated that every one pg of *B. bigemina* DNA represented 15 parasites (66 fg/parasite). The obtained degree of sensitivity was the same as that recorded by Salem et al. (1999) who used the same genus *Babesia* specific primer set designed on the same target under the same amplification parameters. This similarity of sensitivity may ascribed to proper annealing of PCR primer verified from the same target sequence which present in great sequence similarity in both *Babesia* spp. from different geographical regions. Moreover, this sensitivity in *B. bovis* was higher than that recorded by Fahrimal et al. (1992) who used the same target of B. bovis only and found a range from 1 – 10 parasites per 0.5 ml packed erythrocytes. This difference may attributed to that the primer set designed in their study was conserved only to the sequence of *B. bovis* and annealed at 55°C but here the primer set was common for *Babesia genus* with lowering of annealing temperature to 49°C which enhance a wide scale probability of annealing to more templates even with different geographical strains. This data came enclose to Saiki (1989) and Fahrimal et al. (1992) who described that lowering the annealing

temperature may increase the probability of amplification as well as intraspecific sequence divergence of the apocytochrome b gene may be appeared to be minimal in different geographical strains of *B. bovis* and the primer may have general application to all strains of this parasites.

Furthermore, the recorded sensitivity was higher than that recorded by Salem (1998) who detected DNAs equivalent to 200 parasites of B. bovis and 15 parasites of *B. bigemina* per one ml blood. This discrepancy may be due to the difference in the target sequences used from which the PCR primers were designed as well as the abundance of the target copies. A sugesstion was agreed with Jasmer et al. (1990) and Fahrimal et al. (1992) who stated that the extra chromosomal DNA element occurred as multiple copies in each parasite, hence providing multiple templates in the initial PCR with the equivalent of genomic DNA from one parasite as well as different copies of the sequence appeared to be homogenous within a strain. On the other hand, Salem et al. (1999) stated that the increased sensitivity of extrachromosomal DNA-based PCR test over that of ribosomal RNA may be due to, only three ribosomal RNA gene copies were present per genome of both parasites (Reddy et al., 1991 and Calder, 1994) while the extrachromosmomal DNA had greater than 100 copies per parasite genome.

The present work used variable , narrow and uneven serial dilutions (twofold, fivefold, and tenfold) of parasites DNA which corresponding with a narrow panel of parasites concentrations to avoid escaping of a dilution inbetween. This will aid to detect as few as number of parasites represented by low concentration of these DNA. This idea came from the high sensitivity of this common PCR primer set and species specific probes verified from the multiple copies of the apocytochrome b gene in both *Babesia* spp. as previously mentioned (Fahrimal et al., 1992 and Salem et al., 1999).

From the previously mentioned, it was not necessary to change or decrease the annealing temperature during the amplification to increase the chance of primer annealing as done in Salem (1998) who lowerd the annealing temperature of the ribosomal RNA-PCR primer sets than that in Calder et al. (1996). This may be due to the extrachromosomal target DNA was conserved in different geographical strains and the common PCR primer set will be annealed to both parasites in different geographical regions under the same annealed temperature mentioned by Salem et al. (1999).

Concerning the detection of the carrier cattle by PCR following with proper identification of species specific PCR products. Successful

amplification of 78.3 % of DNA in blood samples for *Babesia* spp.using the common PCR primer pair. Moreover, 30.1 % of the sample gave signals at the expected amplified band for *B. bovis* only and 42.2 % for only *B. bigemina* with the corresponding species specific probes. A 6.02 % of the total samples gave strong signals with both probes of *Babesia* spp. reflected a mixed infection. This recorded rate of infections indicated that *B. bigemina* was more higher than B. bovis in Egypt. The achieved result coincided with that of many authors in Egypt (Mohran, 1998 and Salem, 1998). Moreover this rate was nearly the same as that reported by Salem (1998) although he used other target with low number of copies. This may be attributed to the different localities of sampling as well as the threshold of PCR detection.

Generally, when the carrier animal had a very low level of parasitamia or the dilution of DNA was very high to a degree of very faint to invisible PCR bands were shown by electrophoresis (Fig. 2.A & C and Fig. 3.A). A prolonged exposure of the hybridized blotted membrane to the X-ray film at room temperature was necessary to evaluate the threshold of detection of parasite genomic DNA in the samples as well as to enhance the detection of these invisible PCR bands which reflect a proper rate of infections among carrier animals. This suggestion supported by Sambrook et al. (1989) and Calder (1994).

On the other hand, the identification of the common amplified 644 bp band by species specific probes which employs nonradioactive chemiluminescence detection on X-ray film was the simplest , fastest and safe method of detection. A suggestion may be the same as that mentioned by Calder et al. (1996).

Giemsa stained blood smears examined microscopically from all carrier animals showed the parasite only in 2.4%. The detected parasite couldn't differentiated morphologically, but by PCR, the parasite defined as *B. bovis* in both samples. This obtained rate was lower than that recorded by many authors in Egypt (Sakla, 1975; Fadly, 1996 and Mohran, 1998) and slightly higher than that recorded by Salem (1998). These differences may be attributed to the area undergo sampling and the age of animals.

In conclusion, the present investigation demonstrated that PCR assay followed with species specific hybridization detect and differentiate between both *Babesia* spp. at low parasitamias in carrier cattle and has a suitable sensitivity and specificity for discriminating between the infected and unifected animals during importation and exportation. Also to determine whether vaccinated animals become carriers and thus a source of infection for ticks. Moreover, using

apocytochrome b gene in PCR based test was reliable, sensitive and cheaper than the ribosomal RNA because the former require only one PCR reaction for both *Babesia* spp.

REFERNCES

Boom, R.; Sol, C. J. A.; Salimans, M. M. M.; Jansen, C.L.; Wertheim-Van Dillen , P. M. E. and Van der Noordaa, J. (1990): Rapid and simple method for purification of nucleic acids . J. Clin. Microbiol., 28: 495-503.

Bose, R ; Jacobsen , R.H. ; Gales, K.; Waltisbuhl , D.L. and Wright, I.G. (1990): An improved ELISA for the detection of antibodies against *Babesia bovis* using either a native or a recombinant *B. bovis* antigen . Parasitol. Res. , 76 : 648 – 652 .

Buening, G. M ; Barbet , A. F. ; Myler , P. ; Mahan , S. M. ; Nene , V. and McGuire , T. C. (1990): characterization of a repetitive DNA probe for *Babesia bigemina* . Vet. Parasitol. , 36 : 11 – 20 .

Calder, J.A.M. (1994): Improved diagnosis of bovine babesiosis . Ph. D. Thesis, University of Florida , Gainesville , Fl.

Calder, J. A. M. ; Reddy , G. R. ; Chieves , L. ; Courtney ; C. H. ; Littell, R.; Livengood , J. R. ; Norval , R. A. I. ; Smith , C. and Dame , J.B. (1996): Monitoring *Babesia bovis* infections in cattle by using PCR-based tests . J. Clin. Microbiol., 34 : 2748-2755.

Cate, R. L.; Ehrenfels, C. W ; Tizard , R. ; Voyta , J. C. ; Murphy , O. J. and Bronstein, I. (1991): Genomic Southerns analysis with alkaline-phosphatase conjugated oligonucleotide probes and chemiluminescent substrate AMPPD. Genet. Anal. Tech. Appl., 8: 102 - 106.

Chafick, M.L. (1987): Serological tests for the field diagnosis of *Babesia* infection in calves . M. V. Sc. , Thesis , Fac. Vet. Med., Cairo Univ.

El-Allawy, T.A. (1973): Some studies on bovine piroplasmosis in Assiut . M. V. Sc. Thesis , Fac. Vet. Med. Assiut Univ.

Fadly, R.S. (1996): Serological studies on *Babesia* in Behera Province. M. V. Sc., Thesis , Fac. Vet. Med., Alexandria University .

Fahrimal, Y.; Goff, W.L. and Jasmer, D.P. (1992): Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction

amplification of parasite DNA . J. Clin. Microbiol. , 30 : 1374-1379.

Figueroa, J.V.; Chieves , L. P. ; Johnson , G. and Buening , G. (1998): Detection of *Babesia bigemina* infected carriers by polymerase chain reaction amplification . J. Clin. Microbiol. , 30: 2576-2582.

Innis, M. A. ; Gelfand, D. H. ; Sninsky, J. J. and White , T. J. (1990) : In : PCR protocols : A guide to methods and application . San Diego , CA : Academic press .

Jasmer , D. P. ; Reduker , D. W. ; Goff , W. L. ; Stiller , D. and McGuire , T. C. (1990): DNA probes distinguish geographical isolates and identify a novel DNA molecule of *Babesia bovis.* J. Parasitol. , 76 : 834 – 841 .

Joseph, J. T. ; Aldritt , S. M. ; Unnasch , T. ; Puijalon , O. and Wirth, D. F. (1989): Characterization of a conserved extrachromosomal element isolated from the avian malarial parasite *Plasmodium gallinaceum* . Mol. Cell. Biol. , 9 : 3621 – 3629.

Kuttler, K. L. (1988): Worldwide impact of babesiosis . In : Ristic M. (ed.) Babesiosis of domestic animals and man . pp. 1 – 22 , CRC press , Boca Raton , Fla.

Lodish, H.; Baltimore, D. ; Berk , A. ; Zipursy , S. L. ; Matsudaria , P. and Darnell , J. (1995): Organelle Biogenesis: The Mitochondrion , Chloraplast , Peroxisame and Nucleus . In: Molecular cell biology. 3rd ed. pp. 809 – 852 Scientific American books Inc. USA.

Mahoney, D.F. (1969): Bovine babesiois: a study of factors concerned in transmission. Ann. Trop. Med. Parasitol., 63 : 1-14.

Megson, A. ; Inman , G. J. ; Hunt , P. D. ; Baylia , H. A. and Hall , R. (1991): The gene for apocytochrome b of *Theilaria annulata* residues on a small linear extrachromosomal element . Mol. Biochem. Parasitol., 48: 113 – 116 .

Mohran, K.A.E.H. (1998): Control of bovine babesiosis by using methods of biotechnology. Ph. D. Thesis, Fac. Vet. Med., Cairo Univ.

Morzaria , S. ; Katende , J. ; Kairo , A. ; Nene , V. and Musoke , A. (1992): New methods for the diagnosis of *Babesia bigemina* infection . Mem. Inst. Oswaldo Cruz Rio J. , 87 (suppl.): 201 – 205.

Nagati , H. E. (1947): Some new and rare records of piroplasmosis with a list of the species of *Babesia* and *Theilaria* so far recorded from Egypt . Vet. Rec., 59: 145 – 147.

Reddy, G.R.; Charkarbarti, D.; Yowell , C. A. and Dame , J. B. (1991): Sequence microhetrogeneity of the three small subunits ribosomal RNA genes of *Babesia bigemina* expression in erythrocyte culture . Nucleic acids Res. , 19 : 3641 – 3645 .

Saiki, R. K. (1989): The design and optimization of the PCR, p. 7 – 66 In. H. A. Erlich (ed.) , PCR technology, 1989 . Easton publishing, Boca Raton , Fla.

Sakla, A.A. (1975): Studies on ticks in Assiut governorate with special refernce to their role in transmission of parasitic diseases. Ph.D. Thesis , Fac. Vet. Med. , Assiut Univ.

Salem, G.H. (1998): Using of ribosomal-DNA based PCR method for sensitive detection of carrier and mild *Babesial* infections among cattle in Egypt. Assiut Vet. Med. J., 39 (78): 92 – 108.

Salem, G.H.; Liu, X.J.; Johnsrude, J.D.; Dame, J.B. and Reddy, G.R. (1999): Development and evolution of an extra chromosomal DNA-based PCR test for diagnosing bovine babesiosis. Molecular and Cellular Probes, 13: 107 – 113.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Wright, I.G. and Riddles, P.W. (1986): Biotechnological control of tick-borne disease. Report of the FAO Expert consultation on Biotechnology for Livestock production and Health. Rome, Italy, 6 – 10.

Young, A.S. (1988): Epidemiology of babesiosis , pp. 81 – 98. In M. Ristic (ed.). Babesiosis of domestic animals and man. CRC press, Boca Raton , Fla.

LIST OF FIGURES

Fig. 1: (A) Agarose gel electrophoresis of amplified bands of control samples using common PCR primers. Lanes (1 & 6) reference positive control of *B. bovis* and *B. bigemina* (Mexico strain) respectively. Lanes (2, 3 & 4) Egyptian control samples. Lane 5, control PCR kit (720 bp). The black arrow indicated the expected 644 base pair (bp) common PCR band of *Babesia* spp. (Ma) Lambda/Hind III marker (Stratagene Cat # 201109), its bands are 23.1, 9.4, 6.6, 4.4, 2.3, 2 and 0.6 kilobase pair (Kbp) indicated by dashes on the left side from top to bottom respectively. (B): Southern blot hybridization of panel (A) with *B. bovis* specific probe (signals only at lanes 1, 2 & 4). (C): Southern blot hybridization of panel (A) with *B. bigemina* specific probe (signals only at lanes 3 & 6).

Fig. 2: (A) Threshold of detection of *B. bovis* and *B. bigemina* genomic DNAs in serial dilutions by PCR. Results from agarose gel electrophoresis of *B. bovis* (A) and (C) for *B. bigemina*. Southern blot hybridization (with prolonged exposure of the membrane to X-ray film) (B) with nonradioactive *B. bovis* probe to the amplified DNA from serial dilutions of *B. bovis* DNA and (D) with nonradioactive *B. bigemina* probe to the amplified DNA from serial dilutions of *B. bigemina* DNA. Lanes 1 to 10, serial dilutions of genomic DNA of *B. bovis* and *B. bigemina* separately as 100 pg , 50 pg , 10 pg , 5 pg , 1 pg , 100 fg , 50 fg , 10 fg , 5 fg and 1 fg (each / 10 µl DNA) respectively. The intensities of the signals followed that of the amplified bands.

Fig. 3: (A) Ethidium bromide stained gel of PCR extracted from blood of some carrier cattle using the common PCR primer pair of *Babesia* spp. Some amplified bands were clear and other were invisible. Southern blot hybridization of panel A (with prolonged exposure of the membrane to X-ray film) by *B. bovis* probe (B) and with *B. bigemina* probe (C). Strong signals were observed although its amplified bands were invisible. Lanes (2 & 3) showed mixed infections, Lane 4 had only *B. bovis* infection, Lanes (5, 6 & 8) showed *B. bigemina* infection only and Lane (1 & 7) were negative samples.

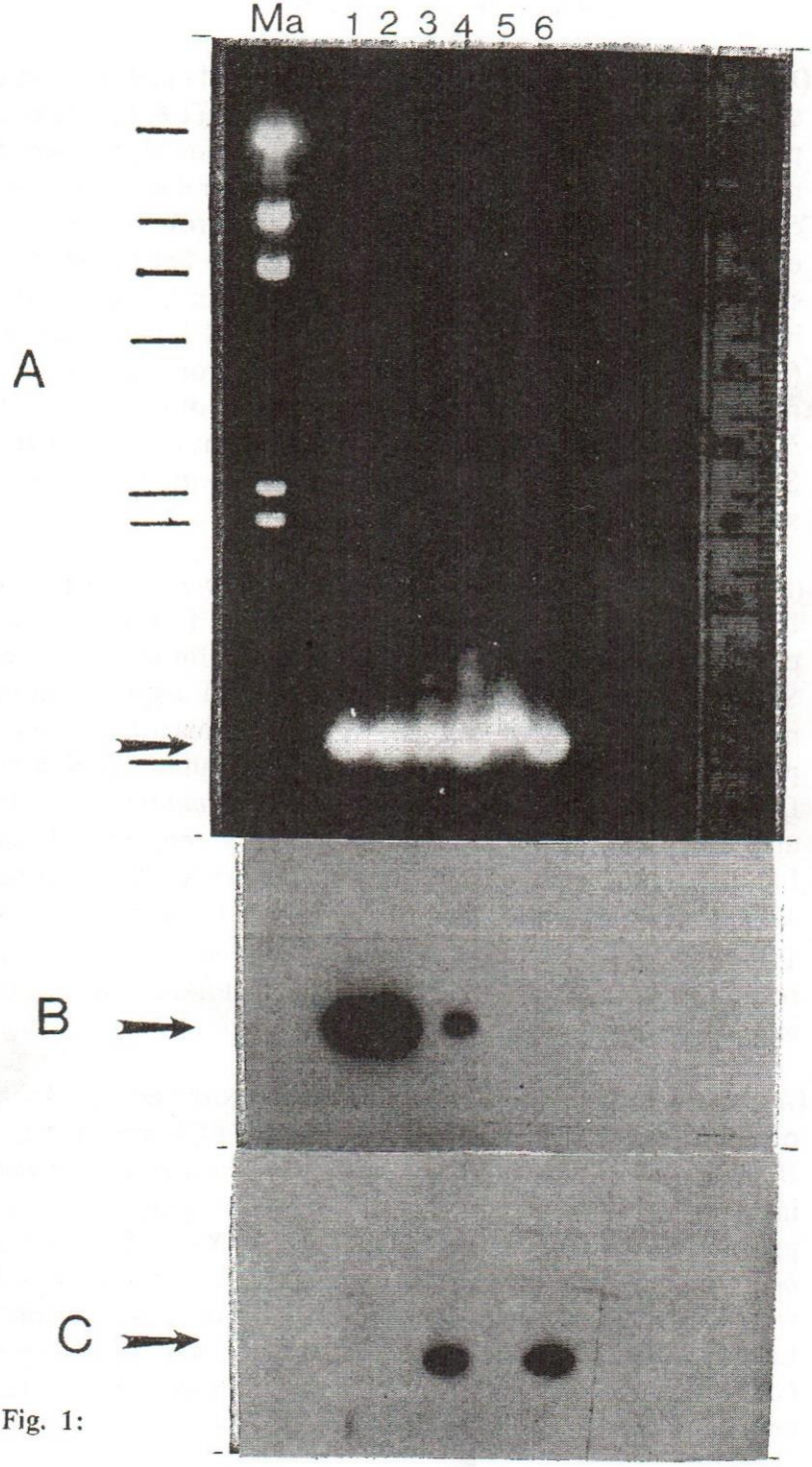

Fig. 1:

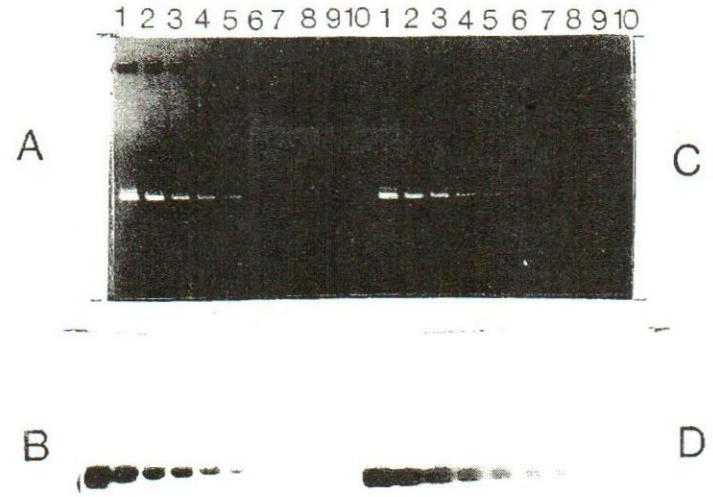

Fig. 2 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10

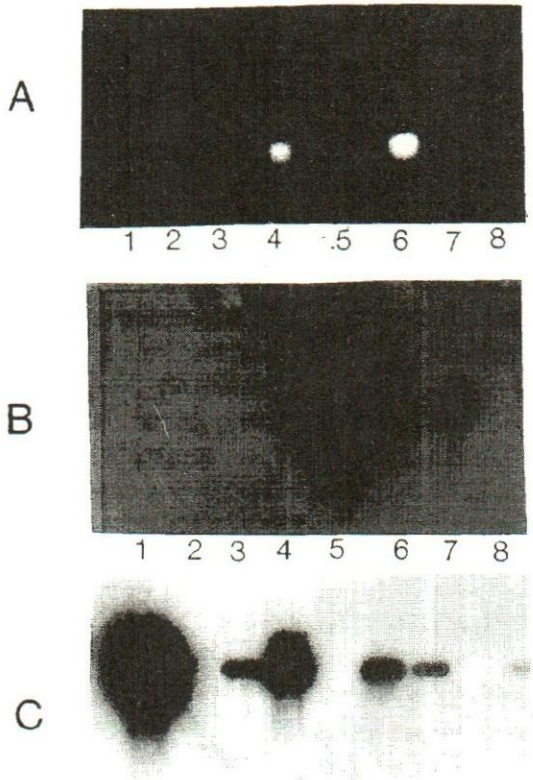

Fig. 3