

Contribution of *Babesia* to the Illness of Cows in Port Said Governorate, Egypt

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Abstract: *Babesia* is an apicomplexan hemoparasite transmitted by ticks to a wide variety of mammalian hosts and cause significant mortality and morbidity to them. This study focused on investigating diseased farm cows (*Bos indicus*) in Port Said governorate in Egypt to briefly estimate the contribution of these pathogens to clinical illness in these economically important animals. Blood samples were collected from 123 ill cows and investigated by routine Giemsa staining and 52 samples were investigated by PCR assay to confirm the infection and detect certain species of *Babesia*. Our results show that the total prevalence of *Babesia* spp. was 13% by routine detection and 23% by PCR assay. *B. bovis* and *B. bigemina* in fections were detected by PCR in 15.3% and 11.5% respectively of the diseased animals. Coinfection with both parasite species was recorded in 3.85 % of animals. This study is the first report of *Babesia* in Port Said governorate and highlights a marked contribution of these parasites to the illness of livestock in Egypt.

Key words: *Babesia* • PCR • Epidemiology • *Bos indicus* • Livestock

INTRODUCTION

Babesia parasites are apicomplexan hemoproteozoans transmitted by Ixodidae ticks [1, 2] and are considered as devastating parasites that affect the production of livestock, mainly cattle and small ruminants [3].

The genus *Babesia* comprises more than 100 species of protozoan pathogens that infect erythrocytes of a wide variety of vertebrate hosts [4] including domestic and wild mammals as well as man [5], however, the major impact occurs in the cattle industry [6]. *Babesia* infections have long been recognized as economically important disease of cattle because they cause extensive erythrocytic lysis leading to anemia, icterus, hemoglobinuria and death [7]. *B. bovis* and *B. bigemina* affect cattle in tropical and subtropical areas imposing a great economic burden on the tropical and subtropical developing countries where raising cattle provides milk and meat as protein sources of high nourishing quality [8, 9].

Detection of these blood parasites is highly beneficial in early diagnosis. Microscopy using Giemsa-stained blood smears has been considered the “gold standard” for detecting *Babesia* and *Theileria* organisms in the blood of animals with acute infections [3]. However, this technique is not suitable for detecting these parasites

in carriers, where the parasitemia is low [10, 11]. Therefore, serological techniques and indirect fluorescent antibody test were proposed for detecting circulating antibodies against these parasites in subclinical infections. However, false-positive and false-negative results due to technical issues and/or to cross-reactions or non-specific immune responses [12] were considered as the major disadvantage of these techniques.

In this study we investigated 123 blood samples from diseased farm cows (*Bos indicus*) in Port Said governorate to briefly estimate the contribution of *Babesia* to clinical illness in these farm animals. 52 blood samples were investigated by PCR assay to confirm the infection and detect selected species of this genus.

MATERIALS AND METHODS

Sample Collection: Diseased cows (*Bos indicus*) that needed urgent clinical treatment by a veterinarian were selected for this study. Blood samples were collected from 123 animals in evacuated tubes containing ethylenediaminetetraacetic acid from the jugular vein. Blood was immediately smeared on clean, dry slides for later staining with Giemsa stain. The remaining blood was frozen until DNA extraction.

Table 1: Oligonucleotides and target genes used for the detection of *Babesia* spp. by PCR.

Primer	Sequence	Target parasite	Target gene (bp)	Reference
B1_F	5'-GTCTTGTAATTGGAATGATGG-3'	<i>Babesia</i> spp.	18S rRNA (411-452 bp)	Cassini et al., 2010
B1_R	5'-TAGTTTATGGTTAGGACTACG-3'			
B3_F	5'-GTGTCAATATGCCTTCGGATTGTT-3'	<i>Babesia bovis</i>	msa2b (680 bp)	Genis et al., 2008
B3_R	5'-GTTATTAAGAGCAGAGAGAACGA-3'			
B4_F	5'-TGTCTCGTTTGCCTTCTAGAGGGACTCCT-3'	<i>Babesia bigemina</i>	SSrRNA (175 bp)	Adham et al., 2009
B4_R	5'-CCGACACGATGCACACTAAACATTACCCAA-3'			

Parasite Staining and Counting: The prepared thin smears were fixed in methanol, stained with Giemsa and microscopically examined at 1000× magnification for parasite detection. Smears were recorded as negative for infection if no parasites were detected in 50 oil-immersion fields [13].

DNA Extraction: 500 µl of whole blood were used for DNA extraction using GeneJET genomic DNA purification kit (K#0721, Fermentas, Germany) according to the manufacturer's instructions.

Oligonucleotides and Polymerase Chain Reaction (PCR): Three pairs of oligonucleotides for detection of *Babesia* spp, *B. bovis* and *B. bigemina* (Table 1) were synthesized in Bioneer Corporation (South Korea) and used in this study. PCRs were performed on 52 DNA samples using Maxima Hot Start PCR master mix kit (#K1051, Fermentas, Germany) according to the manufacturer's instructions. Briefly, 25 µl of Maxima Hot Start Master mix (containing Taq DNA polymerase, dNTPs (400 µM each) and Mg²⁺ (4 mM) were mixed with 4 pmoles of the oligonucleotides, 2 µl of genomic DNA and the reaction was brought to a final 50 µl with nuclease-free water. The reaction was mixed well, spin down and loaded immediately to thermal cycler (Multigene, Labnet, USA) programmed as follows: 4 min at 95 °C for initial enzyme activation followed by 40 cycles of 30 sec at 95 °C for denaturation, 30 sec at the optimized temperature for annealing and 1 min at 72 °C for extension and a final extension step for 10 min at 72°C. PCR products were checked on 1.5% Agarose gels, stained with Ethidium Bromide (Bioshop, Canada) and photographed in a gel documentation system (Photo Doc-IT Imaging system, USA).

RESULTS

Detection of *Babesia* spp. by Microscopy: Blood samples were investigated by the routine Giemsa staining to detect any infection with *Babesia* pathogens and determine their prevalence. Results indicated the infection of 16 animals (13%) with *Babesia* spp.

Detection of *Babesia* spp. by PCR: For a more accurate detection of animal infection with *Babesia* and to determine the prevalence of certain species of these genera, 52 samples were chosen for PCR assay. Using the primer pair (B1) targeting 18S rRNA of *Babesia* spp. PCR successfully amplified a single ~500 bp fragment in 8 samples (15.3%) (Fig. 1, panel A). This product was very close to the expected size (411-452 bp) according to Cassini *et al.* [14]. Some samples, however, resulted in faint PCR products of different sizes ranging from 320 bp to 850 bp (Fig. 1, panel A, samples 6, 7, 8, 9, 11, 13, 14). To further confirm the infection and to identify the *Babesia* species causing it, 2 additional primer pairs were used in a second PCR. One primer pair (B3) targeting the gene *msa2b* was used to identify the samples infected with *Babesia bovis*. As shown in Fig. 1 (panel B), this primer successfully amplified 680 bp fragment of *msa2b* as expected according to Genis *et al.* [15] in 8 samples (15.3%). Unexpectedly, samples 8, 35, 39, 43 and 44 were positive with this primer pair and could not be amplified with the primer pair targeting the 18S rRNA of *Babesia* spp. This finally raises the prevalence of infection of animals with *Babesia* species to 23%. The other primer pair (B4) targeting the SSrRNA gene was used to identify the samples infected with *Babesia bigemina*. As shown in Fig. 1, panel C, this primer pair amplified 175 bp fragment of SSrRNA gene as expected according to Adham *et al.* [16] in 6 samples (11.5%). Out of the 52 samples examined by PCR, 2 samples (3.85%) were coinfecting with both *B. bovis* and *B. bigemina*.

DISCUSSION

In Egypt, there are several tick vectors that can transmit hemoprotozoan parasites to susceptible animals, the most important genera of which are *Hyalomma*, *Boophilus* (*Rhipicephalus*) and *Rhipicephalus* [17]. There is a few number of serological and microscopic studies that provide a limited picture for hemoprotozoan parasites prevalent in Egypt. To our knowledge, there are no previous reports about the spread of *Babesia* parasites in this governorate. It was therefore necessary to assay the

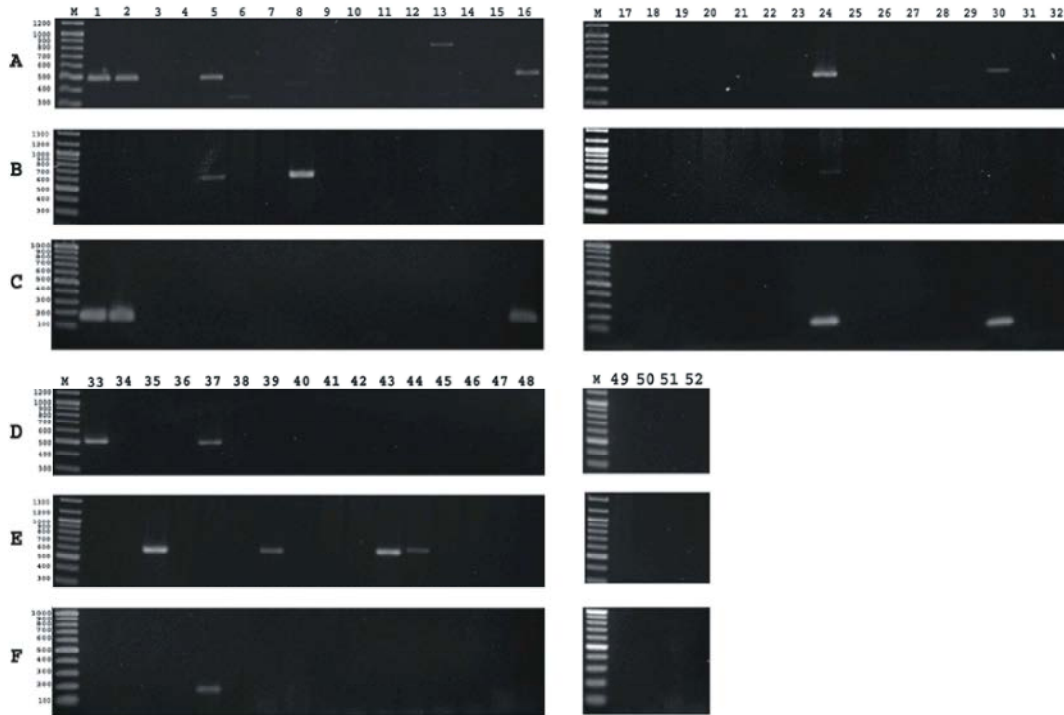


Fig. 1: Results of PCR on 52 DNA samples selected randomly for the detection of *Babesia* spp. (A), *B. bovis* (B), *B. bigemina* (C). Sizes of the expected PCR products are shown in table 1.

prevalence of these tick-borne parasites in this region and to briefly estimate the contribution of *Babesia* to bovine illness.

Microscopic investigation revealed the infection of 13% of animals with *Babesia* spp. and in the randomly selected study sample, PCR revealed the infection of 23% of animals with *Babesia* spp. among which 15.3% were infected with *B. bovis* and 11.5% were infected with *B. bigemina*. Data obtained from microscopic investigation differs a little from those obtained by PCR and this could be related to either the higher sensitivity of the PCR as a detection method, which is now accepted for a wide range of pathogens, or the small sample size selected for PCR. Prevalence of *Babesia* spp. infection in the diseased animals under study is close to *Babesia* infection rates reported previously in other governorates in Egypt. For example, 11.1% of animals were infected with *Babesia* spp. in Quena governorate [18], 8.1% of cattle were infected with *B. bovis* in North Sinai governorate [19], 11.31% of farm animals were infected with *Babesia* spp. in Gharbia governorate [20] and 8.15% of cattle were infected with *Babesia* spp. in Menofia governorate [3]. Some other studies, however, reported higher levels of infection in other governorates; for example El Bahy [21] reported the infection of 65 and 53% of cattle and

buffaloes, respectively, with *Babesia* spp. in Fayom governorate. Fluctuation in the prevalence rates might be due to the variance in animal locale and the timing of study since the latter study was done in the south of Egypt where the environmental conditions (especially temperature and humidity) are strikingly different from those in the current study area. Humidity plays an important role in tick population density, particularly *I. Ricinus* [22, 23]. An increase in temperature may allow vectors to migrate into new areas or to allow a significant development of parasites where sometimes previously, ambient temperatures were too low [24].

To address the discrepancy between the PCR data obtained with the primer pair B1 and the data obtained with the primer pair B3 for samples 8, 35, 39, 43 and 44, the primer pair B1 was blasted to the gene bank. BLAST results showed that this primer pair is 100% identical to and can detect many *Babesia* spp. like *B. bigemina*, *B. microti*, *B. canis*, *B. rossi*, however it can not detect *B. bovis* because the forward primer shows no similarity with any of the *B. bovis* sequences. This was further confirmed by blasting the primer to the piroplasm database which revealed the same result. One more disadvantage of this primer is its ability to amplify *Theileria annulata* and possibly *T. parva*. The traditional

methods of classification must be supported by the recent molecular biological methods, which are useful in differentiating between similar organisms and confirming distinctions based on more subjective characteristics because the same *Babesia* parasite may have different microscopic appearances in different hosts, probably due to host-specific factors, such as splenic function and immunologic predisposition [25]. However, there might be some errors if the primers used in such assays were not verified from time to time parallel to the update occurring in the genome databases, which adds new sequences to the databases.

CONCLUSION

In addition to conventional detection of *Babesia* by microscopy, we applied molecular tools to detect these pathogens in diseased cows. Data showed a marked contribution of these pathogens to clinical illness of farm animals in Port Said. Therefore, *Babesia* should be considered as a danger threatening the life of livestock in Port Said governorate and other governorates of Egypt.

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