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ORIGINAL PAPER

The use of different diagnostic tools for *Babesia* and *Theileria* parasites in cattle in Menofia, Egypt

Mohamed Nayel · Khaled Mohamed El-Dakhly · Mahmoud Aboulaila · Ahmed Elsify · Hany Hassan · Elsayed Ibrahim · Akram Salama · Tokuma Yanai

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Abstract Bovine piroplasmosis is caused by tick-borne hemoprotozoans of the genera *Babesia* and *Theileria* and is the most prevalent in tropical and subtropical countries, causing a major economic impact worldwide. In the current study, a total of 405 cattle of different ages, sexes, and breeds were randomly sampled for surveying and diagnosis of babesiosis and theileriosis using three methods: direct microscopy (blood smears), indirect fluorescent antibody test (IFAT) and polymerase chain reaction (PCR). Giemsa-stained blood smears revealed that, out of 405 examined cattle, 33 (8.15 %) were infected with *Babesia* sp. and 65 (16.05 %) with *Theileria* sp. (total number of infected cattle was 98). Mixed infection was seen in 11 (2.72 %) animals. Moreover, application of the three diagnostic assays on 158 randomly sampled cattle indicated that 17 (10.76 %) and 33 (20.89 %) were positive for *Babesia* and *Theileria* spp. by the direct smear technique, 25 (15.82 %) and 33 (20.89 %) by IFAT (fluorescence was greenish yellow for *Babesia* and yellowish for *Theileria*), and 20 (12.66 %) and 38 (24.05 %) by PCR. Using primers specific for *Babesia* and *Theileria* spp., we found that diagnostic bands appeared at ~350 and ~370 bp, respectively indicating the presence of these piroplasms. Statistically, there was a non-significant difference of the positivity in response to the three techniques; thus, any of these methods can be described as useful for diagnosing blood parasites in both domesticated animals and birds. On the basis of the obtained results, it could be concluded that direct microscopy can be used in acute infections, whereas IFAT and PCR are useful in chronicity.

M. Nayel · A. Elsify · H. Hassan · A. Salama
Department of Animal Medicine and Infectious Diseases,
Faculty of Veterinary Medicine, Menofia University,
Sadat City, Egypt

K. M. El-Dakhly
Department of Parasitology, Faculty of Veterinary Medicine,
Beni-Suef University,
Beni-Suef 62511, Egypt

M. Aboulaila
Department of Parasitology, Faculty of Veterinary Medicine,
Menofia University,
Sadat City, Egypt

E. Ibrahim
Department of Animal Medicine and Infectious Diseases,
Faculty of Veterinary Medicine, Benha University,
Benha, Egypt

K. M. El-Dakhly · T. Yanai (✉)
Department of Veterinary Pathology,
Faculty of Applied Biological Science, Gifu University,
1-1 Yanagido,
Gifu 501-1193, Japan
e-mail: yanai@gifu-u.ac.jp

Introduction

Babesia and *Theileria* species are apicomplexan–hemoprotozoan parasites transmitted by Ixodidae ticks (Preston 2001; Silva et al. 2010) and are viewed to be devastating parasites affecting the production of livestock, mainly cattle and small ruminants. They pose a significant problem for veterinary authorities, occasionally emerging in conjunction with other disease conditions, and thus being difficult to pinpoint (Altay et al. 2008). These infections are of worldwide importance and are characterized by anemia, icterus, hemoglobinuria, and death, and as a result, they have a high economic impact in several parts of the world, including tropical and temperate countries (Wagner et al. 2002).
Bovine babesiosis is caused by multiple species: *Babesia bigemina*, *Babesia divergens*, *Babesia bovis*, and *Babesia major*. *Babesia* species have the potential for wide

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distribution wherever their tick vectors are encountered. Two species, *B. bigemina* and *B. bovis*, have a considerable impact on cattle health and productivity in tropical and subtropical countries. Cattle suffering from theileriosis demonstrate varying clinical signs that range from lymphoproliferative changes with high morbidity and mortality, as seen with *Theileria annulata* and *Theileria parva*, to benign or mild disease, as seen with *Theileria orientalis* (Altay et al. 2008; Safieldin et al. 2011).

Detection of these blood parasites is highly beneficial in early diagnosis. Traditionally, microscopy using Giemsa-stained blood smears has been considered the "gold standard" for detecting *Babesia* and *Theileria* organisms in the blood of infected animals, particularly in acute cases, but not in carriers, where the parasitemia is low, with small numbers of the protozoa in the peripheral blood (Friedhoff and Bose 1994; Bose et al. 1995). Therefore, serological techniques were proposed for detecting circulating antibodies against these parasites, particularly in subclinical infections during epidemiological investigations. In addition, serological diagnosis using the indirect fluorescent antibody test can be used to detect antibodies against the *Theileria* species (Leemans et al. 1997). One disadvantage of such tests is the occurrence of false-positive and false-negative results, involving cross-reactions or improper specific immune response (Passos et al. 1998), while another is the inability to differentiate between previous and current infections, making sensitive and highly specified diagnostic techniques for *Babesia* and *Theileria*. Therefore, the application of PCR-based techniques is imperative in order to detect these hemoparasites in carrier animals (Criado-Fornelio et al. 2009). These methods were used for diagnosis of babesiosis and theileriosis in several species of related countries, showing similar climatic conditions, including Tunisia (M'ghirbi et al. 2008), United Arab Emirates (Jaffar et al. 2010) and Iran (Zaeemi et al. 2011).

The present study was performed for the purpose of surveying and diagnosing both *Babesia* and *Theileria* spp. in cattle in Menofia province, Egypt, using Giemsa-stained blood films, indirect fluorescent antibody test (IFAT)-serological test, and PCR assay.

Materials and methods

Animals and the study area

A total of 405 cattle of different ages, sexes, and breeds were clinically examined for diagnosis of *Babesia* and *Theileria* spp. during field trips in Menofia province (coordinates: 30 °03′00″ N 31 °15′00″ E), Egypt. Animals suffered from signs of blood parasites that were typical indications of babesiosis and theileriosis. Blood smears and blood samples were collected to confirm clinical diagnosis of both diseased and carrier animals.

Blood smears

Thin smears were prepared from EDTA-whole blood on clean and dry slides, fixed in methanol, stained with Giemsa stain, and microscopically examined for the detection of intra-erythrocytic forms of both *Babesia* and *Theileria* spp. piroplasms at 100× objective magnification. The smears were recorded as negative for piroplasms if no parasites were detected in 50 oil-immersion fields (Moretti et al. 2010).

Blood samples

Blood samples were collected for both PCR (with anticoagulant, sodium salt of EDTA) and IFAT (without anticoagulant). Blood was collected from the jugular vein and immediately preserved in Eppendorf tubes containing a few drops of EDTA.

Laboratory assays

Indirect fluorescent antibody test

Available parasite-coated slides for both *Babesia* and *Theileria* antigens were kindly provided by the Veterinary Serum and Vaccine Research Institute, Abassia, Egypt, ready for the indirect fluorescent antibody test as described by Leeflang and Perie (1972). A positive reaction is indicated by a bright fluorescence (Papadopoulos et al. 1996).

Polymerase chain reaction

DNA extraction and amplification DNA extraction was performed according to the Manual Chemical Method (rapid isolation of mammalian DNA) using cell lysis buffer (pH 8.0), ethanol (70 %), isopropanol, potassium acetate solution, red blood cell lysis buffer, and proteinase K (20 mg/ml) as described by Sambrook and Russell (2001).

PCR amplification was performed in a final reaction volume of 50 μl containing 200 μM of each dNTPs, 0.2 uM of each primer, 2.5 U of Taq DNA polymerase (Fermentas, Germany), 10 mM of TBE (tris, boric acid, and EDTA) buffer pH 8.0 containing 1.5 mM $MgCl_2$ and 5 μl of the DNA template. The designated primers were obtained from Bioneer Corporation (064550), Korea. The oligonucleotide sequences of the primers used were forward strand primer BAB GF2 (5′-GTC TTG TAA TTG GAA TGA TGG-3′) and reverse strand primer BAB GR2 (5′-CCA AAG ACT TTG ATT TCT CTC-3′) (Adaszek and Winiarczyk 2008) under the following conditions: an initial denaturation at 95 °C for 5 min followed by 40 cycles of

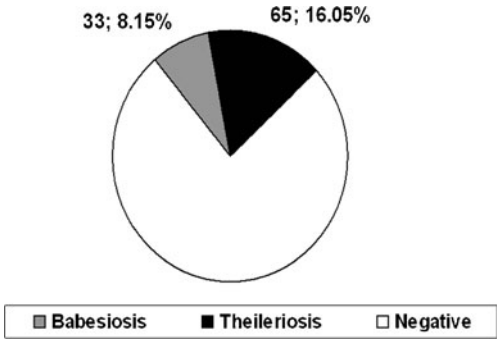

Fig. 1 Diagrammatic scale showing the overall prevalence of *Babesia* and *Theileria* parasites among the examined cattle in Menofia province

denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min.

The amplification reactions were carried out in a PCR thermal cycler Biometra T- personal/Germany S/N 1205334 and the corresponding amplicons were checked on 1.5 % agarose gel, stained with ethidium bromide, examined under UV transilluminator, and photographed using a digital camera.

Statistical analysis

Data were analyzed using multiple comparisons between different diagnostic methods for *Babesia* and *Theileria* parasites, including direct microscopy, IFAT, and PCR using the general linear model, Tukey test. This was carried out using Minitab statistical software (MTW13) (Raza et al. 2007). $P<0.05$ was accepted to be statistically significant.

Results

In this study, parasitological examination of 405 randomly selected cattle, by direct microscopy using Giemsa-stained thin blood films, revealed that 98 had intra-erythrocytic stages of piroplasms of both *Babesia* and *Theileria* spp. with an overall prevalence of 24.2 %. Among those, 33 (8.15 %) were infected by *Babesia* sp., 65 (16.05 %) had *Theileria* sp., and 11 cases showed mixed infection (Fig. 1). These results were consistent with the clinical signs and previous case histories taken from the farmers and owners of animals that were collected from different districts in Menofia province. As a result of chronic infection, the developing antibodies reacted positively using the IFAT, producing a fluorescence that was clearly distinct and greenish yellow (Fig. 2) or yellowish (Fig. 3) in color and indicated the presence of the intra-erythrocytic stages of both *Babesia* and *Theileria* piroplasms, respectively. PCR findings showed that diagnostic bands were produced at ~350 and ~370 bp, and the fragments were specific for both *Babesia* and *Theileria* spp., respectively (Fig. 4). This

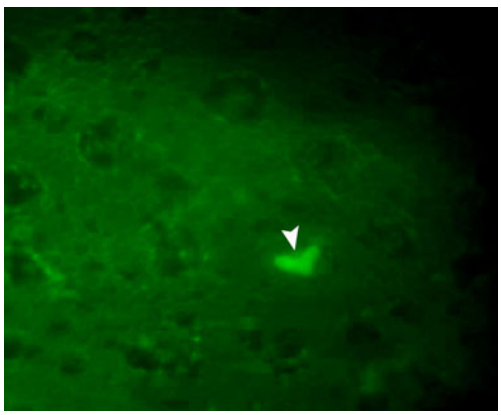

Fig. 2 Greenish yellow fluorescence (*arrow*) indicating intra-erythrocytic stages of *Babesia* sp. piroplasms using IFAT (×100)

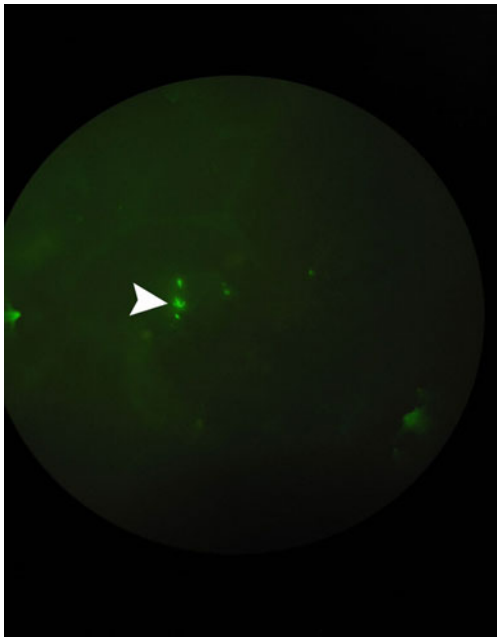

Fig. 3 Yellowish fluorescence of *Theileria* sp. piroplasms (*arrow*) indicating positive reaction with IFAT (×100)

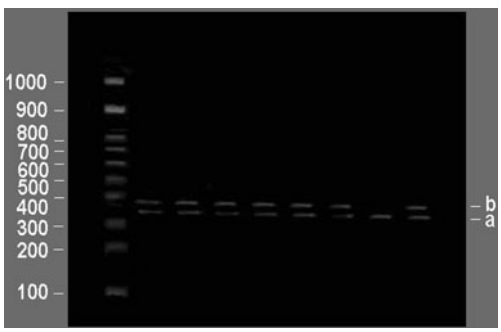

Fig. 4 PCR results showing diagnostic bands at 350-bp for *Babesia* (**a**) and 370bp for *Theileria* (**b**). *Lane M* 100-bp ladder=DNA marker (from Fermentas, Germany)

Table 1 Comparative detection of *Babesia* and *Theileria* parasites in cattle using direct microscopy, IFAT, and PCR

Parasite	Assay (n=158)						*P value*
	Direct microscopy		IFAT		PCR		
	Infected	Percent	Infected	Percent	Infected	Percent	
Babesia sp.	17	10.76	25	15.82	20	12.66	NS
Theileria sp.	33	20.89	33	20.89	38	25.05	NS

finding revealed the presence of both hemoparasites in the blood of examined cattle.

Furthermore, the seroprevalence of *Babesia* and *Theileria* species in cattle using IFAT revealed that out of 158 serum samples examined for the presence of antibodies, 25 (15.82 %) and 33 (20.89 %) were positive for *Babesia* spp. and *Theileria* spp., respectively. Furthermore, PCR findings indicated that 20 (12.66 %) and 38 (24.05 %) were positive for *Babesia* and *Theileria* spp., respectively (Table 1).

The findings in the present study confirmed that the use of direct microscopy, IFAT, or PCR is valuable in the diagnosis of *Babesia* and *Theileria* spp. Based on the confidence intervals calculated using the Tukey method (Minitab), it has been found that the differences between the three methods were not statistically significant, as there was no pairwise variation between infected animals (Fig. 5). This means that in some instances, mainly acute infections, the use of any of these methods may be applicable, although IFAT and PCR are preferable in chronic infections. It is worth mentioning that the methodology in the present investigation is based on collecting a considerable number of animals regardless of studying the effects of sex, age, or season on the infection level.

Discussion

Babesiosis and theileriosis have extensive prevalence and mortality rates with high economic losses in several countries (Shahnawaz et al. 2011). In Egypt, large numbers of cattle are infected with subclinical piroplasmosis (Adham et al. 2009). In such cases, in addition to the parasitological examination of stained blood films for detecting the *Babesia* protozoan parasites, low parasitemia necessitates the use of more advanced diagnostic tools, rather than conventional ones, to detect specific antibodies. In addition, negative microscopic examination does not exclude the possibility of infection (Weiland and Reiter 1988). Subclinical babesiosis and theileriosis lead to the affected livestock, including cattle and small ruminants, becoming chronic carriers of the piroplasms and in turn sources of infection for tick vectors, and cause natural transmission of the disease. Therefore, latent infections are the target in the epidemiology of the diseases.

In the present study, the results obtained from Giemsa-stained blood smears revealed that 33 smears (8.1 %) were positive for *Babesia*. Similar results were obtained by Sevnc et al. (2001) in Turkey and Mazyad and Khalaf (2002) in Egypt. On the other hand, Jeon (1978) in Korea and Osaki et al. (2002) in Brazil detected infection rates of 23 and 64 %, respectively. Fluctuation in the prevalence rates might be due to the variation of environmental conditions that affect both parasites and vectors. For *Theileria* sp., the present investigation showed that 65 (16.05 %) of 405 cattle were positive. These results agreed with those obtained by Jeon (1978) in Korea (17 %), Acici (1995) in Turkey (17 %), and those obtained in Egypt, which included Abu El-Magd (1980) in Quena province (11.1 %) and Adel (2007) in Gharbia province (11.31 %). Conversely, our results opposed a number of reports in Egypt, among them El Bahy (1986), who revealed prevalence rates of 65 and 53 % in cattle and buffaloes, respectively, and Gamal EI-Dien (1993) in El-Behera province, who found that the prevalence of *T. annulata* was 65.4 % using stained blood films.

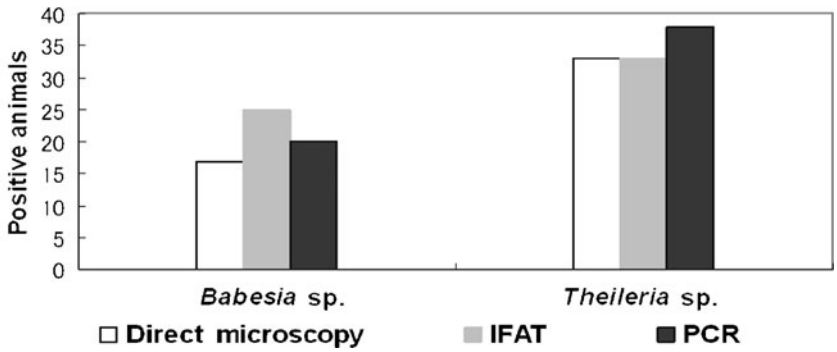

Fig. 5 Comparison of the positivity of *Babesia* and *Theileria* spp. using three diagnostic techniques: direct microscopy, IFAT, and PCR

Variation in prevalence rates could possibly be attributed to an abundance of the vectors as a result of high temperature and humidity. The present investigation showed that mixed infection, using Giemsa-stained blood smears, of *Babesia* and *Theileria* spp. appeared in 11 (2.72 %) of 405 examined cattle. These results coincided with those obtained by Dumanli and Ozer (1987) in Turkey, who found a mixed infection rate for *B. bigemina* and *T. annulata* of 1.5 % in cattle.

Using IFAT, the present study revealed an infection rate of 15.82 % (25/158) for *Babesia* sp. This finding was in accordance with that obtained by Terkawi et al. (2012), who examined a herd of cattle in the central region of Syria using IFAT and found infection rates of 18.36 and 21.74 % for *B. bovis* and *B. bigemina*, respectively. Moreover, Jaffar et al. (2010) in Dubai, UAE recorded infection rates of 10.5 and 33.3 % for equine babesiosis and theileriosis. On the other hand, Singh et al. (2009) recorded a rate of 56.11 % for *B. bigemina* in India, Iseki et al. (2010) noted rates of 68.8 and 75.8 % for *B. bovis* and *B. bigemina* in Thailand, and Sevgili et al. (2010) recorded an infection rate of 43.9 % for *B. bigemina* in Sanliura, Turkey. The variation in infection rates could be due to differences in climatic conditions and species of cattle. Concerning *Theileria* sp., the current investigation revealed that out of 158 bovine serum samples, 33 (20.89 %) was found to be positive for *Theileria* antibodies. This result agreed with that obtained by Sayin et al. (2003), who noted that 34 (21 %) of 155 examined cattle were found to be seropositive for *T. annulata*. Moreover, Adel (2007) in Gharbia province, Egypt reported that the incidence peaks of *T. annulata* seropositive native breed cattle using IFAT were 27.8 and 22 % in spring and summer, respectively. On the other hand, Gamal EI-Dien (1993) in EI-Behera province, Egypt detected that the prevalence of *T. annulata* was 71.9 %, and Caille (1987) in Somalia found that 71.2 % of cattle had antibodies against *T. annulata*. This discrepancy may be due to variation in the susceptibility of the animal species and could also be due to variance in animal locale.

Results of PCR revealed that 20 (12.66 %) of 158 animals were positive for *Babesia* sp. These findings were similar to those mentioned by Oliveira-Sequeira et al. (2005), who recorded a 10 % infection rate for *Babesia* using PCR and M'ghirbi et al. (2008) who revealed an infection rate of 11.11 % for bovine *Babesia* spp. Similar results were obtained by Figueroa et al. (1993) and Gubbels et al. (2002). On the other hand, De Vos and Potgieter (1994) in France found a 20 % infection rate, Costa-Junior et al. (2006) in Brazil found that 8 of 30 cattle (26.7 %) were positive for *Babesia* infection using PCR, and Rania (2009) in Egypt mentioned that the infection rate of *Babesia* sp. was 25.33 %. These discrepancies could be due to changes in locale. The infection rate of *Theileria* using PCR indicated that 60 animals (21.8 %) were positive. This result was similar to those cited by Ogden et al. (2003), Aktas et al. (2005), Altay et al. (2007) and M'ghirbi et al. (2008). Ogden et al. found an infection rate of 23.4 % for *Theileria* sp. in cows in Tanzania using PCR.

The authors concluded that the three techniques, direct microscopy, IFAT, and PCR, are all methods of choice with slight variation in their results, and this was confirmed by obtaining non-significant findings among them. Consequently, they are used in the detection of prevalence of blood parasites, primarily *Babesia* and *Theileria*. Direct microscopy with Giemsa-stained blood films is the conventional and the more practical method on farms, as it is rapid and inexpensive, but it is useful only in acute infections. Moreover, IFAT and PCR are modern assays that help veterinarians detect protozoan parasites in chronic carrier animals, and they circumvent the problem of false negative results obtained under direct microscopy.

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