Title

Seroprevalence of *Babesia bovis*, *B. bigemina*, *Trypanosoma evansi*, and *Anaplasma marginale* antibodies in cattle in southern Egypt

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Abbreviation:

PBS containing 3% skimmed milk, PBS-SM

Abstract

Babesia bovis, B. bigemina, Trypanosoma evansi, and *Anaplasma marginale* infections cause serious diseases in cattle, and are primarily transmitted by arthropod vectors (ticks for *B. bovis*, *B. bigemina*, and *A. marginale* and various types of flies for *T. evansi*). In the last few years, there have been many reports of a high prevalence of certain protozoan infections in northern Egypt, but no accurate or adequate data are available for the southern regions. Therefore, in this study, we screened for evidence of such diseases in economically important cattle species using serum samples. The seroprevalence of protozoan infections in cattle was determined with enzyme-linked immunosorbent assays using species-specific diagnostic antigens. In a total of 301 cattle serum samples, 27 (9.0%), 100 (33.2%), and 127 (42.2%) were positive for specific antibodies against *B. bovis, B. bigemina*, and *T. evansi,* respectively. Sera from 90 cattle were also tested for antibodies against *A. marginale*, and 25 (28%) of them were positive. The highest coinfection rate occurred for *B. bigemina* and *T. evansi* with 10.6% (32/301). When age, sex, locality, and breeding system were investigated as predisposing factors, bulls and cattle < 3 years old were more vulnerable to *B. bovis* infections than older animals, and geographic location affected the *B. bigemina* infection rate. The recorded seroprevalence of hemoprotozoan parasites and *A. marginale* in cattle suggests that these diseases have the potential capacity to detrimentally affect meat and milk production in southern Egypt.

Keywords:

Babesia bovis; *Babesia bigemina*; *Trypanosoma evansi*; *Anaplasma marginale*; cattle; Egypt

1. **Introduction**

Because ixodid ticks are distributed worldwide, babesiosis is the second commonest blood-borne disease of free-living animals (Hunfeld et al., 2008). *Babesia bovis* and *B. bigemina* are the *Babesia* species that most frequently infect cattle, and are commonly transmitted by tick vectors found primarily in tropical and subtropical regions of the world (Chauvin et al., 2009). Babesiosis in cattle is characterized by fever, anemia, and hemoglobinuria (Bock et al., 2004). In Egypt, numerous studies have reported the variable occurrence of *B. bovis* and *B. bigemina* among cattle, particularly studies conducted in the northern regions of the country (Elsify et al., 2015; Mahmoud et al., 2015; Ibrahim et al., 2013; Nayel et al., 2012). *Babesia bovis* and *B. bigemina* DNA was found in *Rhipicephalus annulatus* (formerly *Boophilus annulatus*) ticks in northern Egypt (Adham et al., 2009).

Surra disease, caused by *Trypanosoma evansi*, causes serious economic losses in various types of animals because it affects their fertility and productivity, and *T. evansi* infections of animals are highly endemic in Africa, Asia, and Latin America (Dobson et al., 2009). *Trypanosoma evansi* infections are primarily transmitted mechanically by different types of biting flies, including *Tabanus* and *Stomoxys* species (Birhanu et al., 2015; Salim et al., 2011; Veer et al., 2002; Mihok et al., 1995). These infections are endemic in dromedary camels in Ismailia, northeastern Egypt (Elhaig et al., 2013), and in Cairo (Amer et al., 2011; Haridy et al., 2011). Several studies have also identified *T. evansi* in other animals, such as water buffalo in a Cairo slaughterhouse (Zayed et al., 2010; Hilali et al., 2004), donkeys in Giza (Zayed et al., 2010), and sheep and goats in the Red Sea Governorate (Ashour et al., 2013). Moreover, Veer et al. (2002) demonstrated the role of tabanid flies in the mechanical transmission of *T. evansi*. However, there are few data on the prevalence of *T. evansi* among cattle in Egypt.

Anaplasma marginale, a rickettsial intraerythrocytic pathogen, is host-specific. It infects only ruminants and primarily cattle (Kocan et al., 2010). This parasite is biologically or mechanically transmitted by biting flies and most tick species. The disease caused by *A. marginale* is characterized by fever and general depression, followed by weight loss, progressive anemia, and icterus (Minjauw and McLeod, 2003). Bovine anaplasmosis occurs worldwide, particularly in the USA, Europe, Latin America, Africa, and Asia (Kocan et al., 2010). Cattle infected with *A. marginale* has been reported recently in the Dakahlia Governorate of the delta region of Egypt (El-Ashker et al., 2015) and the DNA of *A. marginale* has been detected in *R. annulatus* and *Hyalomma anatolicum excavatum* in Egypt (Loftis et al., 2006a).

Babesiosis, trypanosomiasis, and anaplasmosis are known to cause serious illnesses in animals (Bal et al., 2014; Mosqueda et al., 2012; Kuttler, 1984), suggesting that they cause a severe financial burden on the livestock industry in northern Egypt. The aim of this study was to determine the seroprevalence of antibodies against *B. bovis, B. bigemina, T. evansi*, and *A. marginale* in cattle in southern Egypt because such data are lacking in this region. We also analyzed various factors predisposing cattle to these diseases, including age, sex, location, and the cattle breeding system used by the farmer.

2. Materials and methods

2.1. Animals and geographic locations

Serum samples from apparently and clinically healthy cattle of different ages, sexes, breeding systems, and locations in Qena and Sohag Governorates in southern Egypt were examined to detect hemoprotozoan and *A. marginale* infections. Serum samples (n = 301) were collected from cattle in the period from May 2014 to June 2015 from the following four groups: group 1, randomly sampled male and female cattle of different ages from breeding systems of individual owners and small-holding farms in different villages in Qena Governorate; group 2, adult cows (over 3 years of age) that were bred in an intensive farming system in Qena Governorate; group 3, adult bulls (over 3 years of age) that were sampled at the Qena slaughterhouse; and group 4, randomly selected cattle of different ages and sexes from the breeding systems of individual owners and small-holding farms from different villages in Sohag Governorate. The details of the various groups of cattle and the geographic locations of the collection sites are shown in Table 1 and Figure 1, respectively. We also analyzed the data by age (less or more than 3 years), sex (bulls or cows), location (Qena or Sohag), and breeding system (individual breeder, small-holding farm, or intensive farming) to identify any factors that might predispose the cattle to infection with *B. bovis*, *B. bigemina*, or *T. evansi*.

2.2. Test animal sera and controls

For the test samples, blood samples were collected from each animal with venipuncture and sera were separated for testing by specific ELISA for each pathogen. For the control samples of *Babesia* species, sera collection and extraction of genomic DNA were performed according to previously described methods (Terkawi et al., 2012). In brief, each blood sample

was added in tubes with and without EDTA anticoagulant to obtain whole blood and sera, respectively. The sera were collected and stored at −20 °C until use. The genomic DNA samples were extracted from the whole blood using a commercial kit (QIAamp DNA Blood Mini-Kit, Hilden, Germany) according to the manufacturer's instructions. The negative (n = 5) and positive (n = 3) control samples were confirmed with Giemsa-stained blood smears (Mosqueda et al., 2012) and a nested PCR analysis (Figueroa et al., 1993) to confirm the parasites and the parasitic DNA, respectively. An indirect immunofluorescence test (Mosqueda et al., 2012) was also used to confirm the *B. bovis*- or *B. bigemina*-specific antibodies. For *T. evansi,* the negative control blood samples (n = 5) or positive controls (n = 3) were collected from cattle in which parasitemia had been tested with a wet blood film examination and mouse inoculation with heparinized blood from such cattle. Anti-*T. evansi*-specific antibodies were also confirmed with a widely used crude antigen-based ELISA, as described previously (Nguyen et al., 2015).

2.3. Heterologous expression of recombinant proteins

In this study, indirect enzyme-linked immunosorbent assays (iELISAs) based on the following recombinant proteins expressed in *E. coli* were used to detect antibodies against bovine babesiosis: the C-terminus of the *B. bigemina* rhoptry-associated protein 1 (BbigRAP-1/CT17) (Kim et al., 2008; sensitivity 96.7%, specificity 93.8%, cut-off value 0.18) and the *B. bovis* spherical body protein 4 (BbovSBP-4) (Terkawi et al., 2011a; sensitivity 96.4%, specificity 96.0%, cut-off value 0.11). Each recombinant protein was expressed with previously described methods, with slight modifications (Terkawi et al., 2011b; Kim et al., 2008). In brief, all the recombinant proteins were expressed as glutathione *S*-transferase (GST) fusion proteins. The purity and quantity of each protein were confirmed by the

visualization of each as a single band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The protein concentrations were measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

2.4. Preparation of crude T. evansi *antigens*

Lysate antigens from the *T. evansi* Tansui strain, which was propagated in HMI-9 medium, were prepared as described previously (Nguyen et al., 2015; Hirumi et al., 1997).

2.5. iELISAs

Purified recombinant antigens (50 µl) at a final concentration of 0.1 µM, or 20 µg/ml for the *T. evansi* crude antigen (Luckins, 2008), were coated onto the wells of ELISA plate (Nunc, Roskilde, Denmark) overnight at 4 °C in the presence of carbonate–bicarbonate buffer (pH 9.6). The plates were washed once with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and then blocked with PBS containing 3% skimmed milk (PBS-SM) for 1 h at 37 °C. The plates were then washed once with PBS-T, and 50 µl of each serum sample diluted 1:100 in PBS-SM was added to the wells. The plates were incubated at 37 °C for 1 h. After the plates were washed six times with PBS-T, they were incubated with horseradish peroxidase-conjugated anti-bovine IgG antibody (Bethyl Laboratories, Montgomery, TX, USA), diluted 1:4,000 for the recombinant proteins or 1:2,500 for the crude antigens with PBS-SM at 37 °C for 1 h. The plates were washed a further six times before 100 µl of substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, 0.3 mg/ml 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); Sigma, St Louis, MO, USA) was added to each well. The optical density at 405 nm (OD_{405}) after incubation for 1 h

at room temperature was measured with an Infinite® F50/Robotic ELISA reader (Tecan Group Ltd, Männedorf, Switzerland). The reading for the GST protein was subtracted from those for the recombinant antigens (BbigRAP-1/CT17 and BbSBP-4). The cut-off values were determined as the mean OD_{405} values plus three standard deviations for the negative control sera used for *B. bovis*, *B. bigemina*, or *T. evansi* (BbigRAP-1a/CT cut-off, 0.05; BbSBP-4 cut-off, 0.08; and *T. evansi* lysate antigen cut-off, 0.15).

2.6. Competitive ELISA to detect A. marginale *antibodies*

Dairy cattle from a farm on which an outbreak of bovine anaplasmosis was suspected 1 year before this study began were checked for the presence of *A. marginale*-specific antibodies. In this study, the sera from these cattle were checked using a commercial *Anaplasma* antibody test kit and competitive ELISA, according to the manufacturer's instructions (Veterinary Medical Research and Development Inc., Pullman, WA, USA). The negative and positive controls provided by the manufacturer were used to validate the test and the results were calculated according to the manufacturer's instructions. The formula used to calculate the percentage (%) inhibition was: $\%I = 100\ (1 - [\text{sample } OD_{405}/OD_{405} \text{ of negative control}\}$. Samples that yielded $\%I < 30\%$ were considered negative, and samples that yielded $\%I \geq 30\%$ were regarded as positive.

2.7. Statistical analysis

The significance of the differences in the incidence rates of the different diseases and risk factors was determined with a χ^2 test. A P value of < 0.05 was considered statistically significant. The 95% confidence intervals were calculated with www.vassarstats.net. χ^2

values and odds ratios were calculated with the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Seroprevalence of hemoprotozoa and A. marginale *in cattle*

In this study, the seroprevalence of *B. bovis* and *B. bigemina* was investigated with iELISAs based on antigens *B. bovis* SBP-4 and *B. bigemina* RAP-1Ct, respectively, which were shown to be effective diagnostic tools in a global survey of babesiosis (Li et al., 2014; da Silva et al., 2013; Ibrahim et al., 2013; Terkawi et al., 2011a, 2011b; Boonchit et al., 2006). We found that 100/301 (33.2%) and 27/301 (9.0%) of the cattle tested were seropositive for *B. bigemina* and *B. bovis* antibodies, respectively (Table 2). These infection rates for bovine babesiosis are similar to those recorded in northern Egypt using a competitive ELISA for *B. bigemina* (32.8%), but lower than that reported for *B. bovis* (21.3%) by Mahmoud et al. (2015). The seropositive rate for bovine babesiosis in our study was also higher than those recorded in Faiyum (15.6%) in central Egypt and in Beheira (10.6%) in northern Egypt. Notably, in this study, we used the same antigens for the iELISA survey as those reported by Ibrahim et al. (2013). Therefore, the discrepancy might be largely related to the different time period and geographic locations used in the present study. In the only other published study, cattle and buffaloes were screened for *B. bovis* and *B. bigemina* infections in Sohag Governorate and the results were negative for all the cattle and buffalo samples tested (Elsify et al., 2015). This finding might be attributable to the small number of animals tested (eight cattle and 14 buffaloes) in the study of Elsify et al. (2015) and the different screening approach they used, which involved the detection of parasitic DNA. In the present study, the significantly higher seroprevalence of *B. bigemina* (33.2%) compared with *B. bovis* (9.0%) might be related to the broader range of *B. bigemina*-transmitting tick species. It has been shown that *R.* (*Boophilus*) *microplus, R. annulatus*, *R. decoloratus*, and *R. evertsi* transmit *B.*

bigemina, whereas *B. bovis* is mainly transmitted by *R. microplus* and *R. annulatus* (Chauvin et al., 2009; Bock et al., 2004).

To the best of our knowledge, there have been no reports from Egypt of the prevalence of *T. evansi* in cattle, whereas its presence in camels, a highly susceptible and severely infected animal species, has been published (Derakhshanfar et al., 2010). Antibodies against *T. evansi* have also been found in water buffaloes (24%) in the cities of Cairo and Giza with serological examinations using a card agglutination test (CATT/*T. evansi*) (Hilali et al., 2004). In our study, using a similar diagnostic method, we observed a high prevalence of *T. evansi* (127/301, 42.2%; Table 2), which might be attributable to the different animal species tested and the location of the study. Another study in the Halaib and Shalateen triangle in the Red Sea Governorate of southern Egypt, which is close to our study areas, showed 100% seropositivity and 100% detection of parasite DNA in camels, sheep, and goats (n = 7 for each species) using both CATT/*T. evansi* and PCR, respectively (Ashour et al., 2013). This result may be attributable to the small number of animals tested and the endemicity of *T. evansi* in this region, which is the main port for camel importation from Sudan, Somalia, and other African countries where *T. evansi* is known to be endemic.

Only the group 2 cattle were screened for antibodies against *A. marginale* because this group had a previous history of suspected anaplasmosis. The total number of *A. marginale*-positive serum samples in this group was 25/90 (28%) (Table 2). Because there is limited information about the presence *A. marginale* in Egypt, it is difficult to compare our results with those of other studies. A recent study using PCR demonstrated that 10% of sick cattle were infected with *A. marginale* in the delta region of Egypt (El-Ashker et al., 2015). Previous surveys of the presence of *A. marginale* DNA in ticks collected from different

animal species and locations showed that only two of 1,019 ticks were positive for *A. marginale* DNA (Loftis et al., 2006a, 2006b).

As shown in Table 3, the most prevalent coinfection was with *B. bigemina* and *T. evansi* (32/301, 10.6%), followed by coinfection with *B. bigemina* and *A. marginale* (7/90, 8%). This result demonstrates the high probability of multiple-pathogen infections in the same animals, suggesting that different vectors are responsible for disease transmission and that the veterinary care of these animals has been seriously defective.

3.2. Risk factor analysis

In the present study, we analyzed age, sex, geographic location, and breeding system as risk factors for infection. The group 1 cattle had the highest seropositive rate for *B. bigemina* (42/100, 42%), whereas the group 4 cattle had the highest seropositive rate for *T. evansi* (38/76, 50%). This indicates that the group 1 and group 4 cattle, which both contain animals of different ages and sexes that were bred on small holdings and individual breeding systems, were the most highly infected groups (Table 2). This finding may reflect the low incomes and education of the animal owners and the use of inappropriate veterinary care for these animals.

A markedly higher prevalence of *B. bovis* was recorded in males (13/61, 21%) than in females (14/240, 5.8%) (odds ratio [OR] 4.4, $P = 0.00015$) (Table S1). This result may indicate that males are more susceptible to infection with *Babesia* because male sex hormones, such as testosterone and estradiol, adversely affect the host's effector immune cells, so infections cause greater parasitemia and anemia, as reported for *B. microti* by Sasaki et al. (2013). Similarly, age was considered a statistically significant risk factor because cattle younger than 3 years exhibited higher infection rates with *B. bovis* (13/61, 21%) than older

cattle (14/240, 5.8%; OR 4.4, P = 0.00015; Table S1). Similar results were reported by Terkawi et al. (2011b), who noted a higher prevalence of *B. bovis* in young animals, supporting our observations. However, our results are not consistent with previous reports that showed significantly higher rates of infection in older animals than in younger ones (Ibrahim et al., 2013; Sukanto et al., 1993). The study by Vannier et al. (2004) indicated that age-related resistance to *B. microti* infection mainly depends on the genetic makeup of the susceptible hosts, and not on their age. In contrast, the effect of location was only apparent for *B. bigemina* infections, for which the prevalence rate was significantly higher in Qena Governorate (91/225, 40.4%) than in Sohag Governorate (9/76, 12%; OR 5.1, P = 0.0001; Table S2). This finding is mainly attributable to the semi-desert landscape and relatively higher environmental temperature in Qena than in Sohag, suggesting more favorable conditions for tick reproduction and development in Qena. In contrast to the seroprevalence of bovine babesiosis, no significant differences were recorded when the same variables (age, sex, location, and breeding style) were examined for *T. evansi* (Table S3). This may be related to differences in the epidemiological patterns of these infections, including the transmitting vector and animal susceptibility.

Although there have been no reports of arthropod vectors in the area we investigated, host-specific vectors have been studied in different regions of Egypt. *Babesia bigemina* has been identified in *R. annulatus* (Adham et al., 2009; El Kammah et al., 2001, El Kammah et al., 2007), and *B. bovis* in *R. annulatus* (Adham et al., 2009). *Anaplasma marginale* was detected in *R. annulatus* and *Hyalomma anatolicum excavatum* in Siwa and Wadi El Natroun, northwestern Egypt (Loftis et al., 2006a). Various species of tabanid flies that are known to transmit *T. evansi* by mechanical means (Birhanu et al., 2015; Veer et al., 2002) have been reported in the Sinai Peninsula in northeastern Egypt (Muller et al., 2012) and in Aswan in

far southern Egypt (Morsy and Habib, 2001). These arthropod vectors may also occur in our region and be responsible for the high prevalence rates of the diseases investigated.

3.3.Critical view of diagnostic methods

ELISAs for the serodiagnosis of *B. bovis*, *B. bigemina*, *T. evansi*, and *A. marginale* infections in livestock offer many advantages in epidemiological studies. rRAP-1/CT is highly specific for *B. bigemina* and SBP-4 for *B. bovis* (Ibrahim et al., 2013; Terkawi et al., 2011a, 2011b; Kim et al., 2008; Boonchit et al., 2006). The specificity of the *A. marginale* antibody test kit was checked and reported by the manufacturer. The crude *T. evansi*-antigen-based ELISA is the reference test recommended by the World Organisation for Animal Health (OIE) for the serodiagnosis of *T. evansi* and is widely used in these types of studies (Nguyen et al., 2014, 2015; Thuy et al., 2012). Therefore, the ELISAs used in this study are highly specific, with few cross-reactions. ELISAs can provide a comprehensive record of disease endemicity in cattle, including subclinical and past infections. However, serodiagnoses cannot differentiate between recent and latent infections.

Premunition, a characteristic phenomenon of bovine babesiosis and anaplasmosis, protects previously infected cattle from the severe forms and complications of subsequent infections (Palmer and McElwain 1995; Kuttler and Johnson 1977). A murine model of *T. evansi* infection has shown that mice infected with this parasite and cured by drugs were considerably more resistant to the infection than those infected with the parasite for the first time (Gill 1971a, 1971b). Therefore, the presence of *T. evansi*-specific antibodies in cattle may not prevent their reinfection with *T. evansi*, but they should afford some degree of immunological resistance to the disease. Consequently, it should be entirely feasible to confirm *T. evansi* endemicity in cattle using both serodiagnostic and pathogen detection

methods. Because we observed a marked seroprevalence of hemoprotozoan parasites and *A. marginale* in cattle in southern Egypt, further examination of these pathogens with PCR and/or microscopy will be required in our next study in this region of Egypt.

4. Conclusions

Bovine babesiosis, trypanosomiasis, and anaplasmosis are associated with severe economic losses in cattle farming. Preventive and therapeutic measures against these diseases are expensive, and untreated cattle infected with these pathogens experience high morbidity, mortality, and culling rates, as well as reduced milk and meat production. High antibody titers are considered indicative of enzootic equilibrium and low risk of outbreaks (Bock et al., 2004). The observed seropositivity values show that the hemopathogens are present in the studied herds but most animals have not been exposed to them, and therefore lack protection. This situation of enzootic imbalance can lead to disease outbreaks. Therefore, infection with hemoprotozoan parasites or *A. marginale* should be considered as risk for the food industry, especially because cattle are very important in meat and milk production in this country. Accordingly, a revision of the current control and preventive measures put in place for the diseases caused by these pathogens should be considered to avoid the economic losses associated with these devastating animal diseases. This study has highlighted the paucity of available data on the prevalence of *B. bovis*, *B. bigemina*, *T. evansi*, and *A. marginale* infections in cattle and the complete lack of information on the vectors responsible for transmitting of these diseases in southern Egypt. Therefore, more research is required to determine the current importance of these diseases in Egypt and augment its contribution to the national income.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure legend

Fig. 1: Geographic distribution of the Egyptian sampling sites used in this study. Dark-colored areas with different letters indicate the governorates that were investigated. 1, Qena; 2, Sohag.

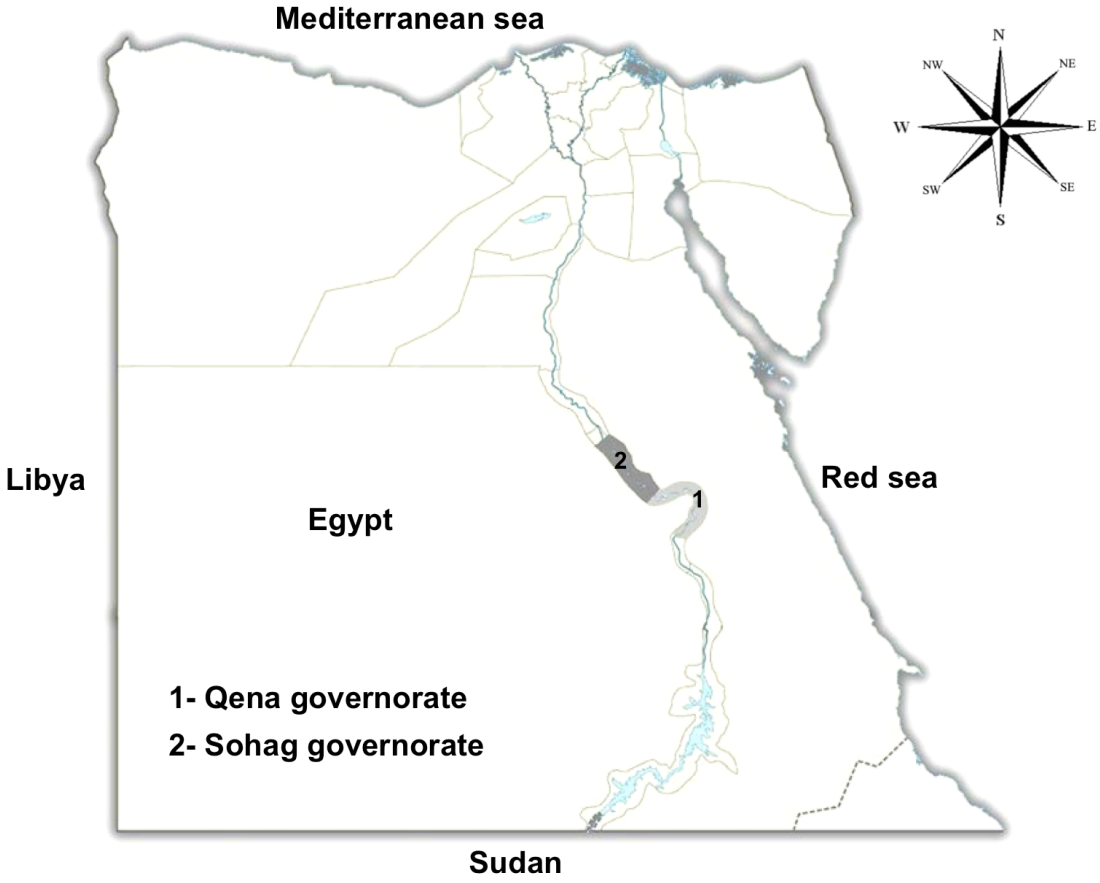

Table 1
Geographic locations and numbers of cattle samples tested in this study

Sampling area	Governorate	Type of breeding system	Number of animals (Total n=301)	Sex	Age
Group 1	Qena	Individual-small holders	100	male, female	mixed
Group 2	Qena	Intensive farming	90	female	> 3 years
Group 3	Qena	Individual-small holders	35	male	> 3 years
Group 4	Sohag	Individual-small holders	76	male, female	mixed

Table 2
Seroprevalence of antibodies against hemoprotozoan parasites and *Anaplasma marginale* in cattle

Sampling area (sample number)	*Babesia bovis*		*Babesia bigemina*		*Trypanosoma evansi*		*Anaplasma marginale*	
	No. of positive (%)	95% CI	No. of positive (%)	95% CI	No. of positive (%)	95% CI	No. of positive (%)	95% CI
Group 1 Qena (n=100)	7 (7)	3.1-14.4	42 (42)	32.3-52.3	43 (43)	33.3-53.3	-	
Group 2 Qena (n=90)	5 (6)	2.1-13.1	35 (39)	28.9-49.8	33 (37)	27-47.5	25 (28)	19.1-38.4
Group 3 Qena (n=35)	12 (34)	19.7-52.3	14 (40)	24.4-57.8	13 (37)	22-55	-	
Group 4 Sohag (n=76)	3 (4)	1-11.9	9 (12)	5.9-21.8	38 (50)	38.4-61.6	-	
Total (n=301)	27 (9.0)	6.1-12.9	100 (33.2)	28-39	127 (42.2)	52-63.4	-	

CI = confidence interval
95% CI calculated according to the method described at http://vassarstats.net/
"-" indicates that this type of infection was not investigated because no data were available for *A. marginale*

Table 3
Mixed infections of blood protozoa and *A. marginale*

Type of pathogen	Sampling area (sample number)				
	Group1 (n=100)	Group 2 (n=90)	Group 3 (n=35)	Group 4 (n=76)	Total positive/test
B. bovis/ B. bigemina	1 (1)*	1 (1)	2 (6)	0	4/301 (1.3)
B. bovis/T. evansi	0	0	1 (3)	0	1/301 (0.3)
B. bigemina/ T. evansi	19 (19)	7 (8)	3 (9)	3 (4)	32/301 (10.6)
T. evansi/ A. marginale	-	6 (7)	-	-	6/90 (7)
B. bigemina/ A. marginale	-	7 (8)	-	-	7/90 (8)
B. bovis/ B. bigemina/ T. evansi	4 (4)	4 (4)	6 (17)	0	14/301 (4.7)
B. bigemina/ T. evansi/ A. marginale	-	4 (4)	-	-	4/90 (4)
Total mixed infections	24 (24)	29 (32)	12 (34)	3 (4)	68/301 (22.6)

*Numbers in parentheses are percentages
"-" indicates that this type of coinfection was not investigated because no data were available for *A. marginale*

Table S1

Risk factors for *B. bovis* in cattle

Cattle group	No. of tested	No. of positive (%)	OR (95% CI)	*P - value*
Age				
< 3 years old	61	13 (21)	4.4 (1.9-9.9)	0.00015
> 3 years old	240	14 (5.8)		
Gender				
Male	61	13 (21)	4.4 (1.9-9.9)	0.00015
Female	240	14 (5.8)		
Location				
Qena	225	24 (10.7)	2.9 (0.8-9.9)	0.076
Sohag	76	3 (4)		
Breeding				
Individual owner /small holders	211	22 (10.4)	2 (0.7-5.4)	0.17
Mass Farming	90	5 (6)		

OR = odds ratio, CI = confidence interval

95% CI calculated according to method described at http://vassarstats.net/

χ^2 test was used to detect the difference between variables.

Table S2
Risk factors for *B. bigemina* in cattle

Cattle group	No. of tested	No. of positive (%)	OR (95% CI)	*P - value*
Age				
< 3 years old	61	19 (31)	0.9 (0.5-1.6)	0.69
> 3 years old	240	81 (33.8)		
Gender				
Male	61	19 (31)	0.9 (0.5-1.6)	0.69
Female	240	81 (33.8)		
Location				
Qena	225	91 (40.4)	5.1 (2.4-10.6)	0.0001
Sohag	76	9 (12)		
Breeding				
Individual owner /small holders	211	65 (30.8)	0.7 (0.4-1.2)	0.17
Mass Farming	90	35 (39)		

OR = odds ratio, CI = confidence interval
95% CI calculated according to method described at http://vassarstats.net/
χ^2 test was used to detect the difference between variables.

Table S3

Risk factors for *T. evansi* in cattle

Cattle group	No. of tested	No. of positive (%)	OR (95% CI)	*P - value*
Age				
< 3 years old	61	29 (48)	1.3 (0.7-2.3)	0.34
> 3 years old	240	98 (40.8)		
Gender				
Male	61	29 (48)	1.3 (0.7-2.3)	0.34
Female	240	98 (40.8)		
Location				
Qena	225	89 (39.6)	0.7 (0.4-1.1)	0.11
Sohag	76	38 (50)		
Breeding				
Individual owner /small holders	211	94 (44.5)	1.4 (0.8-2.3)	0.2
Mass Farming	90	33 (37)		

OR = odds ratio, CI = confidence interval

95% CI calculated according to method described at http://vassarstats.net/

χ^2 test was used to detect the difference between variables.