An epidemiological survey of bovine *Babesia* and *Theileria* parasites in cattle, buffaloes, and sheep in Egypt

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ABSTRACT

Cattle, buffaloes, and sheep are the main sources of meat and milk in Egypt, but their productivity is thought to be greatly reduced by hemoprotozoan parasitic diseases. In this study, we analyzed the infection rates of *Babesia bovis*, *B. bigemina*, *Theileria annulata*, and *T. orientalis*, using parasite-specific PCR assays in blood-DNA samples sourced from cattle (n=439), buffaloes (n=50), and sheep (n=105) reared in Menoufia, Behera, Giza, and Sohag provinces of Egypt. In cattle, the positive rates of *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis* were 3.18%, 7.97%, 9.56%, and 0.68%, respectively. On the other hand, *B. bovis* and *T. orientalis* were the only parasites detected in buffaloes and each of these parasites was only found in two individual DNA samples (both 2%), while one (0.95%) and two (1.90%) of the sheep samples were positive for *B. bovis* and *B. bigemina*, respectively. Sequence analysis showed that the *B. bovis* Rhoptry Associated Protein-1 and the *B. bigemina* Apical Membrane Antigen-1 genes were highly conserved among the samples, with 99.3–100% and 95.3–100% sequence identity values, respectively. In contrast, the Egyptian *T. annulata* Merozoite Surface Antigen-1 gene sequences were relatively diverse (87.8–100% identity values), dispersing themselves across several clades in the phylogenetic tree containing sequences from other countries. Additionally, the *T. orientalis* Major Piroplasm Surface Protein (MPSP) gene sequences were classified as types 1 and 2. This is the first report of *T. orientalis* in Egypt, and of type 2 MPSP in buffaloes. Detection of MPSP type 2, which is considered a relatively virulent genotype, suggests that *T. orientalis* infection may have veterinary and economic significance in Egypt. In conclusion, the present study, which analyzed multiple species of *Babesia* and *Theileria* parasites in different livestock animals, may shed an additional light on the epidemiology of hemoprotozoan parasites in Egypt.

Key words: *Babesia*, Egypt, livestock, PCR, *Theileria*.

1. Introduction

Piroplasmosis caused by different species of *Babesia* and *Theileria* in various wild and domestic animals affects the health status of the infected hosts [1]. Disease outbreaks in livestock animals related to infections with these parasites are of great economic significance. Among the *Babesia* species associated with bovine babesiosis, *Babesia bovis*, *B. bigemina*, and *B. divergens* are considered the most virulent [2]. While *B. bovis* and *B. bigemina* are found in tropical and subtropical regions of world, *B. divergens*, which is also defined as a zoonotic agent, is common in Europe [2,3]. *Babesia* sporozoites released from infected ticks during blood feeding infect the host's red blood cells (RBCs), where they transform into merozoites [4]. The asexual multiplication of merozoites within the RBCs results in hemolysis of the cells, leading to anemia and jaundice in a host animal [2]. The clinical picture associated with *B. bovis* infection includes nervous and respiratory symptoms caused by the sequestration of infected RBCs in the capillary beds of vital internal organs [5].

In cattle, *T. parva* and *T. annulata* are the main etiological agents of severe clinical theileriosis [6], but *T. orientalis*, a benign *Theileria* parasite, has also caused outbreaks of *Theileriosis* in several countries [7,8]. In contrast to most *Babesia* species, *Theileria* sporozoites infect the host leukocytes, where they undergo schizogony and merogony [4,6]. Because *T. parva* and *T. annulata* schizonts induce rapid proliferation of leukocytes, these species are classified as transforming *Theileria* parasites [9]. In contrast, *T. orientalis* does not induce leukocyte proliferation and is therefore referred to as a non-transforming *Theileria* parasite [6]. Merozoites released upon schizont lysis are infective to RBCs [6]. While *T. annulata* and *T. orientalis* merozoites efficiently multiply in RBCs, merogony in RBCs is less pronounced in *T. parva* [6,10]. While *T. parva* is endemic in eastern, central, and southern

Africa, *T. annulata* is common in north Africa, southern Europe, and Asia [11], and *T. orientalis* has a worldwide distribution [7,8,12-18].

Most of the animals that recover from the clinical diseases caused by *Babesia* and *Theileria* parasites remain carriers of these diseases [19,20]. Subclinical infections may also be common among animals that are resistant to clinical piroplasmosis [2]. Detection of carriers and subclinical infections is essential for estimating the level of risk posed by *Babesia* and *Theileria* parasites. Therefore, data from epidemiological surveys could be useful for gauging the efficacy of the parasite control programs implemented in the past. Based on the findings of such surveys, parasite control strategies could be modified where needed. Microscopic examination of Giemsa-stained blood smears is a simple and common method for identifying blood parasites. However, because of low parasitemias at the carrier stage, microscopy may not be an effective diagnostic tool as it lacks sensitivity and specificity [21,22]. Currently, DNA detection techniques, such as PCR assays are preferable for epidemiological investigations, because these methods are specific, sensitive, and capable of detecting active infections [23].

In Egypt, cattle, buffalo, and sheep are the main source of meat, milk, and their related products. Clinical diseases caused by *Theileria* and *Babesia* species are common among the cattle and buffaloes in this country [24-26]. Disease outbreaks often lead to economic losses from reduced productivity, require costly veterinary treatment, and can result in the death of affected animals. Previously, a number of epidemiological studies of *Babesia* and *Theileria* parasites have been conducted in Egypt. *B. bovis* and *B. bigemina* have been reported in cattle, buffaloes, and ticks [27,28]. However, the study areas in these investigations were usually limited to one or two provinces of the country. Furthermore, most of the past epidemiological surveys have focused on either *Babesia* or *Theileria*, but studies aimed at simultaneous detection of both parasites have not been conducted. Additionally, *T.*

orientalis, which has been reported in several countries, has never been studied in Egypt. In the present study, therefore, we conducted an epidemiological survey of *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis*, using blood-DNA samples collected from cattle and buffaloes reared in four Egyptian provinces. Sheep populations were also surveyed to investigate the possible infections with these parasite species.

2. Materials and methods

2.1. Blood sampling and DNA extraction

A total of 594 blood samples were collected from cattle, buffaloes, and sheep during a period from August to October, 2013. In detail, cattle (n=439) were sampled in four different Egyptian provinces (Menoufia, Behera, Giza, and Sohag), while blood samples were collected from buffaloes (n=50) reared in the same provinces, except for Giza (Fig. 1, Table 1). Similarly, sheep (n=105) were sampled in the same provinces, except for Sohag. In Egypt, livestock animals are maintained under three major management practices, intensive, semi-intensive, and extensive systems. Under the intensive rearing system, large herds of exotic animals are kept within proper housing facilities, while cross-bred animals are managed by semi-intensive system. On the other hand, the extensive management is characterized by few numbers of local animals and low production inputs. Cattle in the sampled locations were maintained under intensive, semi-intensive, or extensive management systems, while the buffaloes were reared solely under the extensive system. In contrast, the sheep were managed by semi-intensive or extensive practices. All the animals were apparently healthy during the sampling period. The ages of the sampled animals ranged from 0.5 – 10, 0.5 – 7, and 1 – 5 years for cattle, buffaloes, and sheep, respectively. Blood samples were collected from the tail veins of the cattle and buffaloes, while the sheep blood samples were collected from their jugular veins. Approximately 2 ml of whole-blood was collected from each animal into a Vacutainer tube containing EDTA. The blood samples were labeled and stored at –20 °C, until the DNA extractions were conducted. DNA samples were extracted from 300 µl of the blood samples using a commercial kit (Promega, Madison, WI, USA) following the manufacturer's instructions, and then stored at –20 °C until further use.

2.2. PCR detections of *Babesia* and *Theileria* parasites

Detection of *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis* in the DNA samples from the field-blood samples was conducted using previously described diagnostic PCR assays targeting the Rhoptry Associated Protein-1 (RAP-1), Apical Membrane Antigen-1 (AMA-1), *Theileria annulata* Merozoite Surface Antigen-1 (Tams-1), and Major Piroplasm Surface Protein (MPSP) genes, respectively [29-32]. While *B. bovis* was detected using a nested PCR assay [29], single-step PCR assays were employed to detect the other parasites surveyed [30-32]. Primer sequences and PCR cycling conditions are detailed in a previous report [33]. After gel electrophoresis of the PCR products, the products were ethidium bromide stained and visualized under UV light. Detection of a band similar to that in size of the positive control for a particular species was considered to be a positive result.

2.3. Cloning and sequencing PCR products

For each parasite species, PCR amplicons with high band intensities were extracted from agarose gels using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and then cloned into a plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA). For each amplicon, two clones were sequenced using an AB1 PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.4. Sequence and phylogenic analysis

The nucleotide sequences determined in this study were initially analyzed using the basic local alignment tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/>). The sequence identities among the Egyptian sequences and between Egyptian and homologous sequences from other countries were determined for each parasite species using the EMBOSS NEEDLE software program (<http://emboss.bioinformatics.nl/cgi-bin/emboss/needle>). Phylograms were

constructed using the nucleotide sequences generated in this study together with the homologous gene sequences reported from other countries. An online version of MAFFT software [34] was used to construct the phylogenetic trees, based on the neighbor-joining method [35] with a Jukes–Cantor substitution model [36].

2.5. Statistical analyses

The upper and lower limits of the confidence intervals of the positive rates were calculated for each parasite species using the OpenEpi program (<http://www.openepi.com/v37/Proportion/Proportion.htm>), which is based on a previously described method [37].

3. Results

The species-specific PCR assays detected all parasite species surveyed (i.e., *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis*) in the cattle populations (Table 1). Among the DNA samples sourced from cattle (n=439), 86 (19.6%) were positive for at least one parasite species. In cattle, the most common parasite was *T. annulata* (9.56%), followed by *B. bigemina* (7.97%), *B. bovis* (3.18%), and *T. orientalis* (0.68%). Among the provinces surveyed (Fig. 1), Behera had the highest positive rates for three parasite species, *B. bovis* (8.51%), *T. annulata* (29.78%), and *T. orientalis* (6.83), while *B. bigemina* was common in Giza (13.33%). Additionally, *T. orientalis* was detected only among the cattle population in Behera. By contrast, all eight of the cattle sampled in Sohag were negative for all the parasites surveyed. Only *B. bovis* or *T. orientalis* were detected in the buffalo-derived DNA samples, but their rates were low (both 2%), and each parasite species was detected in two individual animals from Menoufia. Similarly, in Behera one and two sheep were positive for *B. bovis* and *B. bigemina*, respectively. Mixed infections were limited to cattle, in which eight such animals harbored two parasite species. Among them, four samples were infected with *B. bovis* and *T. annulata*, while four others were positive for *B. bovis* and *B. bigemina*, *B. bovis* and *T. orientalis*, *B. bigemina* and *T. annulata*, or *T. annulata* and *T. orientalis*.

Among the positive samples, 13, 12, 14, and four of the PCR amplicons sequenced were *B. bovis* (11 cattle, one buffalo, and one sheep), *B. bigemina* (10 cattle and two sheep), *T. annulata* (14 cattle), and *T. orientalis* (three cattle and one buffalo), respectively. Out of these 43 PCR amplicons, the nucleotide sequences of both of the clones were identical to each other for 25, while the sequences of remaining 18, including 1 *B. bovis* *RAP-1*, 3 *B. bigemina* *AMA-1*, and 14 *T. annulata* *Tams1*, differed between the two clones. Therefore, a total of 61 sequences were registered in GenBank, for which the accession numbers

AB917246 - AB917306 were obtained. The *B. bovis*, *B. bigemina*, and *T. orientalis* nucleotide sequences of the PCR amplicons were of the expected sizes (298, 211, and 776 bp, respectively), while the *T. annulata* sequences were 771 (AB917275-AB917298), 777 (AB917299), or 783 (AB917300-AB917302) bp in length. The identity values among the nucleotide sequences of *B. bovis* *RAP-1* determined in this study (AB917246-AB917259) ranged from 99.3 to 100%. Additionally, the *RAP-1* sequences derived from a buffalo (AB917258) and a sheep (AB917259) were identical to each other and to some of those derived from cattle (AB917246-AB917251, AB917253-AB917255, and AB917257) in Egypt. Furthermore, the *RAP-1* sequences from Egypt shared 99.0–99.3% sequence identity with a previously published sequence from Sri Lanka (AB845432). The high identities among the Egyptian *RAP-1* sequences are reflected in the phylogenetic tree, in which they were found in a single clade (clade 2) (Fig. 2).

The identity values among all 15 Egyptian *B. bigemina* *AMA-1* sequences (AB917260-AB917274) ranged from 95.3 to 100%, while the values of those from cattle (AB917263-AB917274) and sheep (AB917260-AB917262) ranged from 97.2 to 100% and 97.6 to 100%, respectively. Additionally, the sheep-derived *AMA-1* sequences (AB917260-AB917262) shared 95.3–100% identity values with the *AMA-1* sequences (AB917263-AB917274) from the Egyptian cattle. Moreover, the *AMA-1* sequences from this study also shared 97.6–100% identity with a sequence from South Africa (KF626604). All of the Egyptian sequences were found in a single clade of the *AMA-1* gene-based phylogeny (clade 2) (Fig. 3).

For *T. annulata*, the Egyptian *Tams-1* sequences shared 87.8–100% identity with each other and 96.0–100% with a sequence (AF214819 in clade 3) (Fig. 4) from Mauritania. Additionally, the *Tams-1* sequences from this study shared 89.9–99.6% identity with a sequence (KJ021627 in clade 1) previously reported in Egypt. In the phylogenetic analysis,

the Egyptian sequences were dispersed across all the clades, with the exception of clade 4 (Fig. 4).

The identities among the four *T. orientalis* *MPSP* sequences from Egypt (AB917303-AB917306) ranged from 87.1 to 100 %. While two cattle-derived (AB917303 and AB917304) and one buffalo-derived (AB917306) sequence were classified as *MPSP* type 2, a single sequence from cattle (AB917305) was identified as *MPSP* type 1 (Fig. 5). Among the *MPSP* type 2 sequences, the cattle derived sequences (AB917303 and AB917304) were closely related to a sequence from China (AB571981, type 2) (99.4 and 99.9% sequence identity, respectively), while that from buffalo (AB917306) shared a high identity value (97.8%) with a sequence from Australia (AB520947, type 2). Additionally, the *MPSP* type 1 sequence from Egypt (AB917305) is close to a Chinese *MPSP* sequence (HQ322621), showing 99.7% identity.

4. Discussion

Bovine piroplasmosis caused by *Babesia* and *Theileria* parasites has a worldwide distribution and inflicts a severe economic burden on farming communities [1]. In this study, cattle, buffalo, and sheep populations, which were bred in different geographical locations in Egypt, were surveyed for bovine parasites. Subsequently, the genetic diversity of each parasite species, based on the gene sequences determined from the PCR amplicons generated by species-specific primers, was studied.

Our findings indicate that the most common blood parasite in cattle is *T. annulata*. Although clinical cases of *T. annulata* are common in Egyptian bred cattle and water buffaloes [24-26,38], there are no studies detailing the detection of *T. annulata* from infected carrier animals. Therefore, our study should provide useful preliminary data to the policy makers associated with animal production in Egypt that could allow them to adapt better informed control strategies against this potentially harmful pathogen. Sequencing and phylogenetic analyses of *T. annulata* showed that *Tams-1* sequences are diverse in Egypt. Genetic diversity among *Tams-1* sequences is very common because of intragenic recombination, which occurs within the tick vectors [39]. Further studies aimed at understanding the relationships between genetic diversity and the biological behaviors of *T. annulata* are of interest as such investigations have not been conducted.

The second and third most common parasites in cattle were *B. bigemina* and *B. bovis*, respectively, and the rates of infections in these animals agree with those estimated in a previous study [27]. In the phylogenetic analyses, the *B. bovis* *RAP-1* and *B. bigemina* *AMA-1* sequences were detected in single clade of their respective trees. However, the gene fragments analyzed in this study are relatively conserved among field isolates from different geographic locations [40,41]. Therefore, analyzing suitable marker genes, such as merozoite

surface antigen genes in *B. bovis*, might provide a better picture of the genetic diversity in *B. bovis* [42-45], while identification of suitable genetic markers are essential for *B. bigemina*. The present study described *T. orientalis* for the first time in Egypt, and found that the parasites detected belonged to two different allelic types of *MPSP*; types 1 and 2. Scant literature exists on the epidemiology of *T. orientalis* in Africa. Although this parasite has been reported in Burundi, its diagnosis was based on morphological and antigenic properties [46]. Gebrekidan et al. (2014) [47] described *T. orientalis* in Ethiopian cattle, based on *18S rRNA* sequences. Additionally, a single *MPSP* sequence (AB016278) from Kenya falls into the *MPSP* type 3 clade [48]. Therefore, the detection of different *MPSP* types in Egypt may prove useful for furthering our understanding of the population structure of *T. orientalis* in Africa. Although the movement of *T. orientalis*-infected animals and ticks across the territorial borders might be a possible reason for the presence of this parasite in several African countries, analysis of *T. orientalis MPSP* types in the this region is essential to confirm this assumption. Among the *MPSP* genotypes, type 2 is thought to be relatively virulent, as this type is commonly associated with clinical outbreaks and more anemic in cattle than the other genotypes [7]. Furthermore, detection of *MPSP* type 2 in buffaloes is particularly significant, because this allelic type has never been reported to occur in buffaloes. Thus, it appears that the involvement of buffaloes as reservoir hosts for the *MPSP* type 2 should not be ruled out. Nevertheless, the presence of *MPSP* type 2 in Egypt could indicate that the cattle populations in this country are vulnerable to clinical theileriosis as a result of infection with *T. orientalis*.

On a per district basis, Behera province had the highest parasite-positive rates in cattle among all the surveyed provinces. The humid climate in Behera, which may favor the survival of tick populations, is a possible explanation for the high rates of infection. However, additional studies to determine the population structures of the ticks in the different Egyptian

provinces are needed to confirm this assumption. However, the small sample size of the animals surveyed in Sohag might explain why none of the parasites of interest were detected in this region.

The rates of infection with *Babesia* and *Theileria* in buffaloes and sheep in this study are low. Although some studies have described tropical theileriosis in Egyptian buffaloes [25,26], *T. annulata* was not detected in the buffaloes screened herein. It is possible that the small sample size is related to the low parasite-positive rates seen in the buffaloes. Interestingly, in this study, the DNA samples from sheep were infected with *B. bovis* and *B. bigemina*, although sheep are not a known host for these parasites. It is noteworthy that similar findings were observed in a previous study, in which *Theileria equi* and *Babesia canis* were detected in dog and horse, respectively [49]. Similarly, *B. bigemina* infection was reported in goats from Vietnam [50]. Although the pathobiological significance of host shifting is not known, it might be one of the survival strategies used by hemoprotozoan parasites.

In conclusion, our study detected *Babesia* and *Theileria* species in cattle, buffaloes, and sheep in several provinces of Egypt. For the first time, *T. orientalis* was reported in cattle and buffaloes bred in this country. These findings have economic significance and indicate the importance of introducing effective prevention and control strategies throughout Egypt to minimize the prevalence of bovine hemoprotozoan parasites, such as those investigated in this study.

Acknowledgments

We thank Dr. Amer Hamada (Faculty of Veterinary Medicine, Sadat City University) and Ms Hiroko Yamamoto (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary medicine) for their excellent technical assistance. We also thank the owners and staff of the study farms in Egypt. This study was supported by grants from the Scientific Technique Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, from the Japan Society for Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, and from the JST/JICA, Science and Technology Research Partnership for Sustainable Development (SATREPS). Ahmed Elsify was supported by a grant from Ministry of Higher Education, Egypt.

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Figure legends

Fig. 1. Sampling locations in Egypt. Blood samples were collected from animals reared in Menoufia (M), Behera (B), Giza (G), and Sohag (S), as indicated by bullet points.

Fig. 2. Phylogenic tree of the *B. bovis* *RAP-1* gene. The neighbor-joining tree [35] was constructed based on the Jukes-Cantor substitution model [36] using the MAFFT program [34]. The nucleotide sequences determined in this study are shown in boldface type letters. Bootstrap values are provided at the beginning of each branch. Note that the Egyptian *RAP-1* sequences are found in a single clade.

Fig. 3. Phylogenic tree of the *B. bigemina* *AMA-1* gene. The neighbor-joining tree was constructed based on the Jukes–Cantor substitution model using the MAFFT program. The nucleotide sequences determined in this study are shown in boldface type letters. Bootstrap values are provided at the beginning of each branch. Note that the Egyptian *AMA-1* sequences are found in a single clade.

Fig. 4. Phylogenic tree of the *T. annulata* *Tams1* gene. The neighbor-joining tree was constructed based on the Jukes-Cantor substitution model using the MAFFT program. The nucleotide sequences determined in this study are shown in boldface type letters. Bootstrap values are provided at the beginning of each branch. Note that the Egyptian *Tams-1* sequences are found in multiple clades.

Fig. 5. Phylogenic tree of the *T. orientalis* *MPSP* gene. The neighbor-joining tree was constructed based on the Jukes–Cantor substitution model using the MAFFT program. The

sequences determined in this study are shown in boldface type letters. Bootstrap values are provided at the beginning of each branch. Note that the Egyptian *MPSP* sequences fall into *MPSP* type 1 and 2 clades.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Table 1. PCR detection of *B. bovis*, *B. bigemina*, *T. annulata*, and *T. orientalis* in cattle, buffaloes, and sheep

Animal type	Provinces	No. of Samples	<i>B. bovis</i>		<i>B. bigemina</i>		<i>T. annulata</i>		<i>T. orientalis</i>	
			No. positive	%(CI ^a)	No. positive	%(CI)	No. positive	%(CI)	No. positive	%(CI)
Cattle	Menoufia	354	9	2.54 (1.34 - 4.76)	27	7.62 (5.2 - 10.87)	24	6.77 (4.59 - 9.88)	0	0
	Behera	47	4	8.51 (3.36 - 19.93)	4	8.51 (3.36 - 19.93)	14	29.78 (18.65 - 43.98)	3	6.83 (2.19 - 17.16)
	Giza	30	1	3.33 (0.59 - 16.67)	4	13.33 (5.31 - 29.68)	4	13.33 (5.31 - 29.68)	0	0
	Sohag	8	0	0	0	0	0	0	0	0
	(Total)	439	14	3.18 (1.90 - 5.28)	35	7.97 (5.78 - 10.89)	42	9.56 (7.15 - 12.68)	3	0.68 (0.23 - 1.99)
Buffalo	Menoufia	33	1	3.03 (0.53 - 15.63)	0	0	0	0	1	3.03 (0.53 - 15.32)
	Behera	3	0	0	0	0	0	0	0	0
	Sohag	14	0	0	0	0	0	0	0	0
	(Total)	50	1	2.00 (0.35 - 10.49)	0	0	0	0	1	2.00 (0.35-10.49)
Sheep	Menoufia	25	0	0	0	0	0	0	0	0
	Behera	27	1	3.70 (0.65 - 18.25)	2	7.40 (2.05 - 23.37)	0	0	0	0
	Giza	53	0	0	0	0	0	0	0	0
	(Total)	105	1	0.95 (0.16 - 5.19)	2	1.90 (0.52 - 6.68)	0	0	0	0

^a 95% confidence interval

*Graphical abstract

Sampling locations in Egypt

Phylogeny of *T. orientalis* MPSP