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RESEARCH ARTICLE

Molecular Confirmation of *Trypanosoma evansi* and *Babesia bigemina* in Cattle from Lower Egypt

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ABSTRACT

Trypanosomosis and babesiosis are economically important vector-borne diseases for animal health and productivity in developing countries. In Egypt, molecular epidemiological surveys on such diseases are scarce. In the present study, we examined 475 healthy and 25 clinically diagnosed cattle from three provinces in Lower Egypt, for *Trypanosoma* (*T.*) and *Babesia* (*B.*) infections using an ITS1 PCR assay that confirmed *Trypanosoma* species presence and an 18S rRNA assay that detected *B. bigemina.* Results confirmed *Trypanosoma* spp. and *B. bigemina* presence in 30.4% and 11% individuals, respectively, with eight animals (1.6%) being co-infected with both hemoparasites. Subsequent type-specific PCRs revealed that all *Trypanosoma* PCR positive samples corresponded to *T. evansi* and that none of the animals harboured *T. brucei gambiense* or *T. brucei rhodesiense*. Nucleotide sequencing of the variable surface glycoprotein revealed the *T. evansi* cattle strain to be most closely related (99% nucleotide sequence identity) to strains previously detected in dromedary camels in Egypt, while the 18S rRNA gene phylogeny confirmed the presence of a unique *B. bigemina* haplotype closely related to strains from Turkey and Brazil. Statistically significant differences in PCR prevalence were noted with respect to gender, clinical status and locality. These results confirm the presence of high numbers of carrier animals and signal the need for expanded surveillance and control efforts.

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INTRODUCTION

Vector-borne diseases represent a major threat to farm animals in tropical and subtropical countries, including Egypt. Diverse *Babesia* and *Trypanosoma* species are known to infect cattle (Bilgiç *et al.,* 2013; Sivakumar *et al.,* 2013), but these have not been fully evaluated in Egypt. In particular, although *Trypanosoma* (*T.*) *evansi* has previously been reported in dromedary camels from Egypt (Amer *et al.,* 2011; Elhaig *et al.,* 2013), to date there has been no confirmation of this species in cattle from Egypt, although *T. brucei* subspecies, *T. brucei brucei*, *T. brucei gambiense* and *T. brucei rhodesiense*, have been reported from cattle and arthropods in neighboring Sudan (Mohammed *et al.,* 2010; Salim *et al.,* 2011; Dyary *et al.,* 2014).

Trypanosoma evansi is transmitted mechanically by hematophagous flies (tabanids and stomoxes), causing production losses, anemia, weight loss and abortion in a range of domestic species in Africa, Asia and South America. This trypanosomosis, also referred to as 'surra', can be fatal in the absence of treatment (Desquesnes *et al.,* 2013) and sub-clinical cases, attributable to chronic infection and/or lower strain virulence (Elhaig *et al.,* 2013), complicating diagnosis and control the disease. Although it is not considered a zoonosis (OIE Terrestrial Manual, 2012), a recent human *T. evansi* infection has been reported in Egypt (Haridy *et al.,* 2011), however, there is a need to evaluate zoonotic potential (Desquesnes *et al.,* 2013).

Babesia bigemina is transmitted by infected *Rhipicephalus* ticks and clinically is characterized by fever, anemia and hemoglobinuria (Tavassoli *et al.,* 2013). As with *T. evansi*, animals that have recovered from acute infection can become carriers and serve as a potential source of infection (Sharma *et al.,* 2013; Ismael *et al.,* 2014). Because of the similarity of clinical symptoms of

vector-borne diseases, clinical examination of infected animals is not sufficient (Bilgiç *et al.,* 2013) and a range of laboratory techniques including blood smears, serology, mouse inoculation and molecular techniques are required to make a definitive diagnosis (Berlin *et al.,* 2012). Although ELISA can be highly effective for epidemiological surveys and for detecting carrier animals, it lacks the ability to differentiate between past and active infections. Babesiosis and trypanosomosis are readily diagnosed by microscopic examination of stained blood smears, but this technique has a low sensitivity in subclinical or chronic infections (Elhaig *et al.,* 2013; Takeet *et al.,* 2013). Molecular techniques in addition to being capable of detecting active infections, offer higher sensitivity and specificity than other diagnostic techniques and are increasingly being used (Almeria *et al.,* 2001; Elhaig *et al.,* 2013; Sharma *et al.,* 2013; Takeet *et al.,* 2013; Tran *et al.,* 2014). We therefore employed molecular methods in this initial assessment of the haemoparasite status of 500 cattle sampled from 20 farms within three adjacent provinces of Lower Egypt.

MATERIALS AND METHODS

Study area and sample collection: A total of 500 blood samples from cattle (110 females and 390 males, ranging from 8 months to 7 years in age) were collected from 20 small scale cattle farms in three provinces (Ismailia, Sharkia and Qalubiya) in the Delta area of Egypt. Many of the farms sampled between March 2013 and April 2014 occurred in close proximity to camel farms and had husbandry practices typical of small-scale rural farms. All animals were clinically examined and signs of fever, oedema in limbs, emaciation and red urine were recorded. EDTA blood samples (10 ml) were collected from 25 clinically confirmed and 475 apparently healthy animals and transported on ice to the laboratory of parasitology, Faculty of Medicine, Suez Canal University.

DNA extraction and PCR amplification: DNA was extracted from whole blood using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). For PCR amplification of multiple pathogenic *Trypanosoma* species in a single reaction, a generic ITS1-PCR (Njiru *et al.,* 2005) was initially performed. The Samples positive for the *T. brucei* subgroup (consisting of *T. evansi*, *T. brucei brucei*, *T. brucei gambiense* and *T. brucei rhodesiense*) by ITS1-PCR were subjected to further rounds of PCR using species-specific primers to distinguishing *T. evansi* (Masiga *et al.,* 1992), *T. brucei gambiense* (Radwanska *et al.,* 2002a) and *T. brucei rhodesiense* (Radwanska *et al.,* 2002b). PCR amplification of a 689 bp fragment of *Babesia bigemina* was performed with the small subunit ribosomal RNA primers (Ellis *et al.,* 1992). All primers used in the present study are listed in Table 1. All PCR reactions were performed in a final volume of 50µl containing 25µl of Go Taq® Green Master Mix 2X (Promega Co. USA), 0.4µM (1µL) of each primer and 3µL of DNA template. Double-distilled water was added to bring the final volume to 50µl. Positive and negative controls were included in all assays. Thermal cycling conditions for *Trypanosoma* species were consisted of an initial 2 min incubation at 95°C, followed by 35 cycles of denaturation at 95°C for 30sec, annealing at the primer-specific temperatures (Table 1) for 30sec and extension at 72°C for 1 min, and a final elongation step at 72°C for 5min. For the *B. bigemina*, PCR cycling conditions were an initial cycle at 95°C for 5min, 30 cycles of 95°C for 45sec, 55°C for 1min and 72°C for 1.5 min, and finally one cycle at 72°C for 5min. PCR products were analyzed by 1.5% agarose gel electrophoresis and photographed using a gel documentation system (Biospectrum UVP, UK).

Nucleotide sequencing and phylogenetic analysis: The PCR product of samples positive for *T. evansi* and *B. bigemina* were selected and sequenced using the variable surface glycoprotein (VSG) (Urakawa *et al.,* 2001) and small subunit ribosomal RNA (18S rRNA) (Ellis *et al.,* 1992) targets, respectively. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and products were outsourced for automated DNA sequencing (ABI 3730XL, Solgent Co. Ltd., South Korea). BlastN searches (www.ncbi.nlm.nih.gov/blast) were performed in order to identify 18S rRNA and VSG gene entries in the GenBank database, with the highest nucleotide sequence identities. An 18S rRNA gene phylogeny was inferred using a range of phenetic and cladistic approaches (Fig. 2). The obtained sequences reported in this study were deposited in the GenBank with the accession number KF726106 (*T. evansi*) and KM076937 (*B. bigemina*).

Statistical analyses: Chi-squared tests were performed (http://vassarstats.net/) to evaluate statistical differences in PCR infection rates of cattle across different provinces, sex and ages classes, and clinical status (Table 2).

RESULTS

Clinical examination: During the study period there were no reports of large-scale surra or babesiois outbreaks among livestock populations in Egypt, although surra was reported in camels from Ismailia and babesiosis had previously been reported in cattle from Menofia, Beheira and Faiyum.

Table 1: Summary of primers used to assess haemoparasite genome presence in this study

Species	Primer sequences (5'- 3')	Annealing temperature (°C)	Gene target	Product size (bp)
Trypanosoma spp.	ITS1 CF: CCGGAAGTTCACCGATATTG ITS1 BR: TGCTGCGTTCTTCAACGAA	58	ITS	250-700
T. evansi	TBR1: GAA TATTAAACAATGCGCAG TBR2: CCATTTATTAGCTTTGTTGC	60	Minisatellite DNA	164
T. brucei gambiense	Sense: GCTGCTGTGTTCGGAGAGC Antisense: GCCATCGTGCTTGCCGCTC	60	Glycoprotein	308
T.brucei rhodesiense	F: ATAGTGACAAGATGCGTACTCAACGC R: AATGTGTTCGAGTACTTCGGTCACGCT	68	SRA gene	284
T. evansi	F: GCCACCACGGCGAAAGAC R: TAATCAGTGTGGTGTGC	58	Rotat 1.2 VSG	480
B. bigemina	Bg3: TAGTTGTATTTCAGCCTCGCG Bg4: AACATCCAAGCAGCTAHTTAG	55	Small subunit ribosomal RNA	689

Table 2: PCR detection of *Trypanosoma evansi* and *Babesia bigemina* in cattle from three Lower Egypt Provinces

Factor		*N*	*Trypanosoma evansi* (%)	*Babesia bigemina* (%)	Co-infection † (%)
Clinical status	Symptomatic	25	12 (48)	9 (36)	3 (12)
	Asymptomatic	475	140 (29.5)	46 (9.6)	5 (1.1)
	χ^2 value	-	3.85*	16.8*	18.1*
Locality	Ismailia	120	32 (26.7)	30 (25)	3 (2.5)
	Sharkia	270	95 (35.2)	25 (9.3)	5 (1.9)
	Qalubiya	110	25 (22.7)	0	0
	χ^2 value	-	6.77*	38.4*	2.5
Age	≤1 year	88	32 (36.4)	10 (11.3)	3 (3.4)
	>1 year	412	120 (29.1)	45 (10.9)	5 (1.2)
	χ^2 value	-	1.8	0.04	2.2
Sex	Female	110	42 (38.2)	13 (11.8)	2 (1.8)
	Male	390	110 (28.2)	42 (10.7)	6 (1.5)
	χ^2 value	-	4.03*	0.09	0.04
Total		500	152 (30.4)	55 (11)	8 (1.6)

† Co-infection: *Trypanosoma evansi* and *Babesia bigemina*; * P<0.05.

Detection of *T. evansi* and *B. bigemina* by PCR assays: Out of 500 blood samples examined by ITS1-PCR, 152 (30.4%) were found positive for *Trypanosoma* species. Subsequent testing by PCR assays specific to *T. evansi, T. brucei gambiense* and *T. brucei rhodesiense*, showed that all 152 positive samples were infected with *T. evansi*. While none of the infections could be ascribed to *T. brucei gambiense* or *T. brucei rhodesiense* on the basis of PCR. The *B. bigemina* PCR assay identified 55 positive animals of which eight samples (1.6%) were co-infected with *T. evansi*.

Clinical status, age, sex and sampling locality: The overall PCR based hemoparasite prevalence was 39.8%, with significantly higher levels of infection being detected in symptomatic animals (P≤0.05). Clinically affected animals had infection rates of 48% and 36%, respectively, for *T. evansi* and *B. bigemina*, whereas rates of 29.5% and 9.6% were recorded in asymptomatic animals, respectively. Co-infection rates were also higher in symptomatic animals (12%) than in carriers (1.1%). The locality with the highest prevalence of *T. evansi* was Sharkia province (35.2%, n=95), followed by the Ismailia province (26.7%, n=32) with Qalubiya having the lowest prevalence (22.7%, n=25). *Babesia bigemina* was only detected at two localities, namely Ismailia (25%, n=30) and 9.3% at Sharkia (23%, n = 25). Co-infections rates for these localities were 2.5% and 1.9%, respectively. Although infection rates were higher in animals younger than one year for individual and mixed infections, these differences were not significantly different (Table 2). However, females had significantly higher *T. evansi* infection rates (38.2%) than males (28.2%).

Nucleotide sequence and phylogenetic analysis of *T. evansi* and *B. bigemina*: The nucleotide sequence of *T. evansi* (GenBank Accession Number KF726106) revealed a 99% sequence identity to a *T. evansi* sequence from a dromedary camel from Egypt (JX888091), and to a strain from India (JX134605), over the 473 nucleotides Rotat1.2 VSG region characterized. Among Egyptian *T. evansi* isolate in the present study and those reported by Amer *et al.* (2011) in Egypt, sequences showed 1-4 bp substitutions compared to reference sequence (JX134605; Fig. 1). Due to the excessively hypervariable nature of *Trypanosoma* VSG, for which inter-specific pair wise divergences exceed 62% on amino acid level, phylogenetic analyses were not performed.

The *B. bigemina* sequence (GenBank Accession Number KM076937) from Egypt was found to represent a unique haplotype, and to have the highest nucleotide sequence identity to EF458198 (from Brazil), differing at three positions from this sequence and at four positions from EF459199 (from Turkey). All methods of analyses confirmed the close relationship of the Egyptian *B. bigemina* strain to haplotypes from Turkey and Brazil, with high levels of nodal support (72–99%; Fig. 2).

DISCUSSION

In Egypt, *T. evansi* and *B. bigemina* are enzootic in camels (Elhaig *et al.,* 2013) and cattle (Ibrahim *et al.,* 2013), respectively. Despite sustained efforts to reduce these infections high seroprevalence to *T. evansi* has been reported in a range of domesticated species, including water buffaloes (Hilali *et al.,* 2004), sheep and goats (Ashour *et al.,* 2013), but not for cattle from Lower Egypt. In the current study in which we assessed cattle reared in close proximity to dromedary camel farms previously shown to be positive for *T. evansi*. We confirmed bovine trypanosome presence in 152 animals using a generic ITS1-PCR capable of detecting multiple pathogenic *Trypanosoma* species in a single PCR (Njiru *et al.,* 2005; Salim *et al.,* 2014). This corresponds to a prevalence of 30.4% for *T. brucei* subgroup species which is higher than the 3.3% prevalence reported in Sudan (Salim *et al.,* 2014). *Trypanosoma evansi* species designation was subsequently confirmed using a species-specific PCR (Masiga *et al.,* 1992) and by nucleotide sequencing of VSG. Although the presence of *T. evansi* in cattle was not surprising, these results represent to our knowledge, the first record of *T. evansi* in cattle from Egypt. Additional species-specific assays indicate the absence of *T. brucei rhodesiense* and *T. brucei gambiense* in the 500 cattle assessed. The detection of *T. evansi* in subclinical animals suggests long endemicity in the studied areas and a high numbers of chronic cases. These asymptomatic carriers constitute a source of infection, not only to cattle but possibly also to animal owners (Takeet *et al.,* 2013), and is of significance as it is often fatal if untreated (Desquesnes *et al.,* 2013).

The prevalence of *B. bigemina* in the current study (11%) was higher than the 5.2% reported previously by Ibrahim *et al.* (2013) and lower than the 32.4% reported by Mahmoud *et al.* (2015) for other provinces of Egypt. These regional differences in prevalence may be due to

differences in husbandry, tick distribution, detection methods, and time of sampling in Egypt. Differences in animal management explain the absence of *B. bigemina*-positive samples in Qalubiya governorate as cattle in this governorate are kept in small numbers, are well cared by their owners, being regularly treated with Diazinon compound that controls ticks (Ghosh and Nagar 2014).

The higher *T. evansi* prevalence compared to *B. bigemina* in this study concurs with the previous studies from other regions, e.g. nine districts of Punjab, India, Northern Kerala, South India and Cholistan Desert, Pakistan and may be related to higher prevalence of tabanid flies, their interrupted feeding patterns and their ability to travel longer distances, in comparison to ticks (Nair *et al.*, 2011; Sharma *et al.*, 2013; Tehseen *et al*., 2015).

Co-infection rates of 12% and 1% in symptomatic and healthy animals, respectively, concur with Sharma *et al.* (2013) who reported the dual infection of both parasites in clinical cases as well as in latent carriers. These and other authors (Sharma *et al.*, 2013; Sivakumar *et al.*, 2013) have reported that the severity of trypanosomosis increases with co-infection with *B. bigemina* or other blood pathogens, compared to monotypic infections. Thus, the detection of co-infections in Egypt, even at low levels, is of clinical relevance.

The significantly higher *Babesia* and *Trypanosoma* infections in young cattle (≤1 year) compared to adults (>1 year) are in agreement with those of Takeet *et al.* (2013) and Terkawi *et al.* (2011) who reported higher trypanosome infection rates in young animals. Similarly, our findings of a slightly higher *B. bigemina* prevalence in females (11.8% versus 10.7% in males), is in agreement with those of Terkawi *et al.* (2011) and the significantly higher prevalence of *T. evansi* in females, in our study, can be attributed to stress factors of pregnancy and lactation, which may increase their susceptibility to infection (Bhutto *et al.*, 2010; Tehseen *et al*., 2015).

Despite high sequence similarity among *T. evansi* isolates, sequence alignment of partial VSG gene of Egyptian isolates revealed 1- 4 bp substitutions as reported earlier (Amer *et al.*, 2011; Elhaig *et al.*, 2013), these less sequences heterogeneity reflect the effect of geography and hosts (Amer *et al.*, 2011). In addition, the existence of gene encoding RoTat 1.2 VSG (Urakawa *et al.*, 2001), is a diagnostic tool to differentiate *T. evansi* from *T. brucei* sugbgroup.

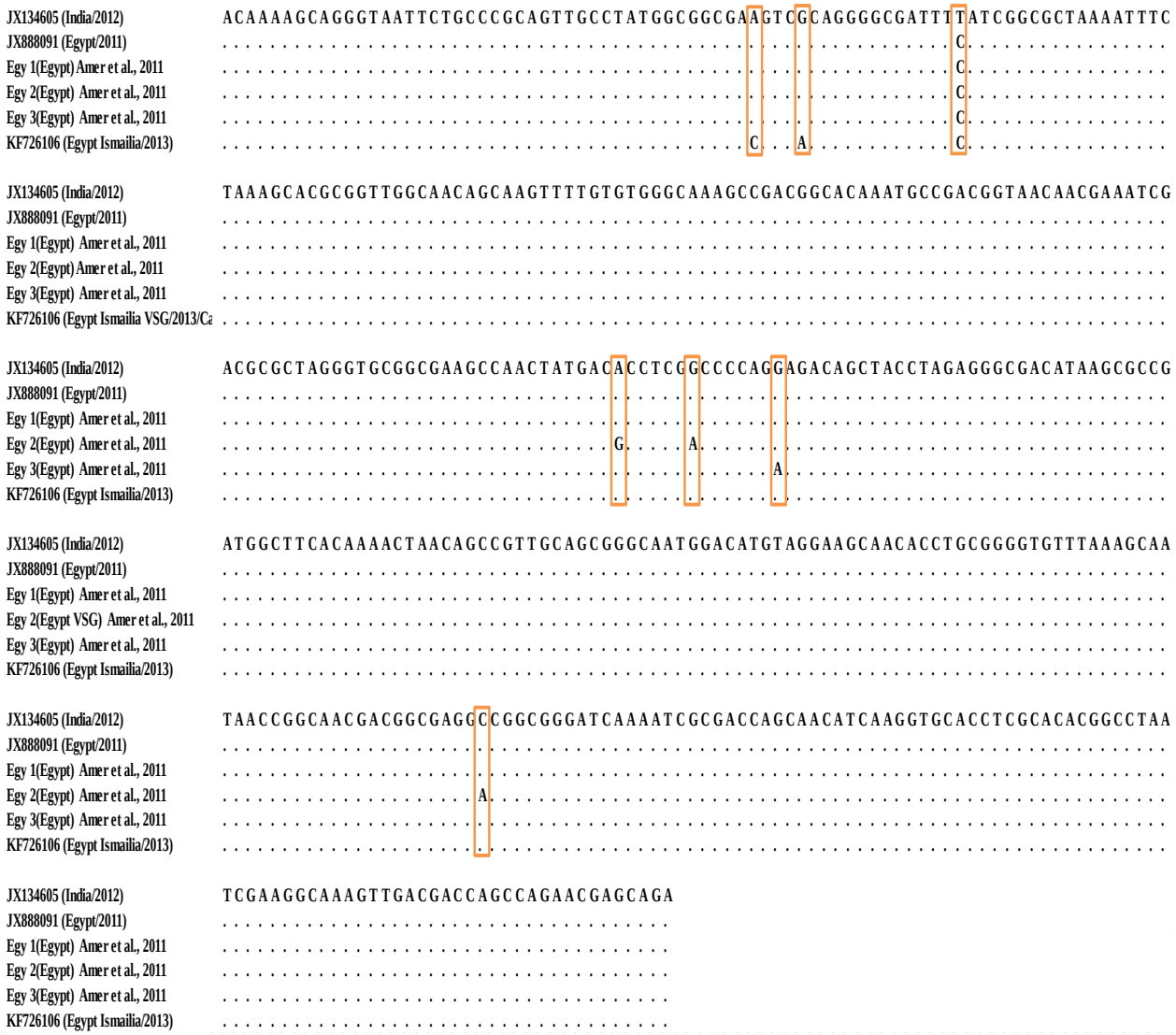

Fig. 1: Nucleotide sequence alignment of *T. evansi* VSG gene variants from Egyptian cattle *T. evansi* (KF726106) with other VSG *T. evansi* from Egypt and reference strain from India (JX134605). Identical bases are shown as dots and sites of variation are shown in boxes.

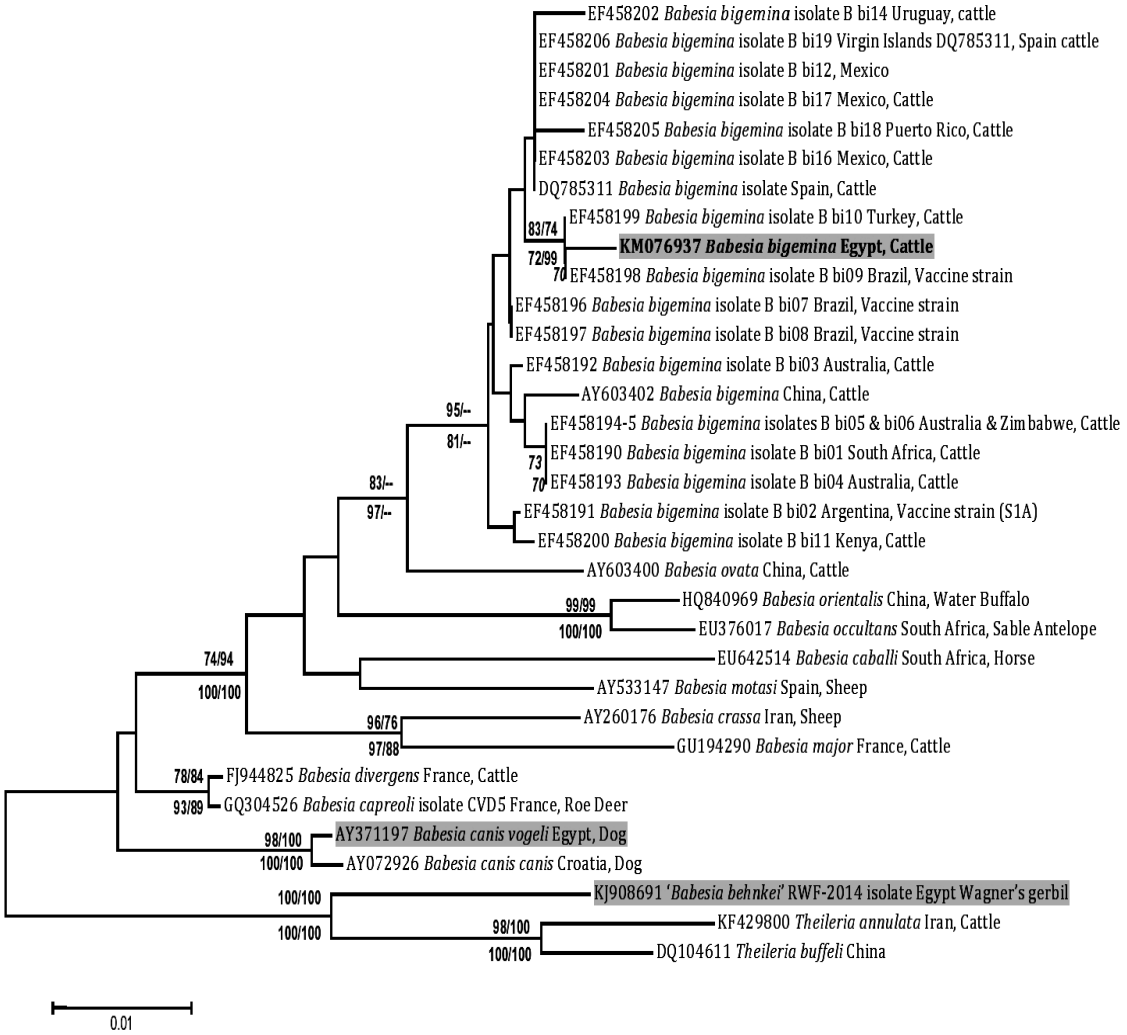

Fig. 2: Minimum Evolution (ME) tree depicting the 18S rRNA gene relationships of Egyptian *Babesia* and *Theileria* strains (shaded grey) and reference strains. The *B. bigemina* cattle strain characterised in this study is indicated in bold. Nodal support values > 70 from ME (10,000 replicates), Maximum Likelihood (ML; 2,500 replicates) and Maximum Parsimony (MP; 1000 replicates) analyses, and posterior probabilities expressed as percentage from the Bayesian Inference (BI) analysis are indicated ME/ML (above the branch) and MP/BI (below the branch). Nodes with high levels of support from the ME analysis alone are denoted in italix.

The *Babesia* 18S rRNA gene phylogeny (Fig. 2) confirmed the close relationship of the Egyptian *B. bigemina* strain to haplotypes B bi 10 (from Turkey) and B bi 9 (from Brazil), with high levels of nodal support (72 – 99%). This sequence constitutes a unique haplotype and the first reference sequence for this species from Egypt. Characterization of additional strains and gene regions would be valuable in future for understanding the genetic diversity among Egyptian *B. bigemina* isolates. Although it was not possible to infer a phylogeny using Rotat 1.2 VSG sequences, our results showed a close relationship between Egyptian cattle *T. evansi* and that isolated from camels in Egypt (99.5%) and to strains from India and Kenya.

Conclusions: This first report of *T. evansi* in cattle in the Egypt reared in close proximity to camel farms has highlighted the high number of sub-clinical infections in three provinces of Lower Egypt. Co-infection of *T. evansi* and *B. bigemina*, is of clinical relevance as are the regional differences in infection rates which highlight the need for further broad-scale epidemiological investigations to ensure that appropriate prevention and control measures are identified and applied. Phylogenetic analysis of 18S rRNA gene sequence of *B. bigemina* isolate of Egyptian origin revealed a close relationship to global isolates from Turkey and Brazil. There is no sequences information for any 18S rRNA gene of *B. bigemina* available from Egypt other than the sequence reported here. It is essential to perform further studies on additional 18S rRNA gene sequences to provide further characterization of *B. bigemina* isolates from Egypt.

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Authors' contributions: MME planed and designed the study. AS, MMM and EKE assisted in data collection, laboratory work and drafting of the manuscript. All authors have read and approved the submission of the manuscript.

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