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Molecular Prevalence and Risk Factors Analysis of Asymptomatic Infection with *Anaplasma marginale* and *Theileria annulata* in Egyptian Cattle

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Abstract

Awareness of the epidemiology of *Anaplasma marginale* (*A. marginale*) and *Theileria annulata* (*T. annulata*) in the asymptomatic carriers is integral for developing efficient control in tropical and subtropical countries. The endemic status of both pathogens among asymptomatic carrier cattle in Egypt is poorly investigated. This study was conducted to detect the prevalence of *A. marginale* and *T. annulata* infections in apparently healthy cattle from six Egyptian provinces based on specific polymerase chain reaction (PCR) assays and to assess its associated risk factors. Two hundred seventy-eight blood samples were collected from cattle in 24 farms and four abattoirs in Beheira, Damietta, Cairo, Fayoum, Qena, and Luxor. DNA was extracted, and *A. marginale msp4* and *T. annulata tams-1* genes were amplified using Real-Time PCR assays. The overall prevalence of *A. marginale* and *T. annulata* were 39.6% and 5.8%, respectively. Meanwhile, the prevalence of co-infection was 2.5%. The prevalence of *A. marginale* (0.0% - 65.1%) and *T. annulata* (0.0% - 25.0%) greatly varied between provinces. Statistical analysis of risk factors indicated that female cows (49.7%; OR = 3.4; p = < 0.0001), Mixed breed (47.4%; OR = 3.6; p = 0.03), dairy (48.2%; OR = 3.7; p = < 0.0001), and small-size farms (60.2%; OR = 4.6; p = < 0.0001) showed a significantly higher risk for *A. marginale* compared to the references. Meanwhile, for *T. annulata*, cattle from Luxor (25.0%; OR = 10.3; p = 0.03) and ages < 2 years (16.7%; OR = 4.6; p = 0.038) had a significantly higher risk against the references. These findings highlight the endemic status of *A. marginale* and *T. annulata* in asymptomatic cattle and analyze the risks influencing their prevalence in Egyptian cattle.

Keywords: *Anaplasma marginale*; *Theileria annulate*; Asymptomatic; Prevalence; Risk factors; Cattle

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1. Introduction

Tick-borne pathogens (TBPs) threaten the global livestock industry (Jongejan and Uilenberg, 1994). The economic losses of TBPs in cattle herds are of significant importance due to the decrease in productivity, cow mortality, expensive veterinary services, and tick control (Lew-Tabor and Valle, 2016). Bovine anaplasmosis, the disease caused by *Anaplasma marginale* (*A. marginale*), and bovine theileriosis caused by *Theileria annulata* (*T. annulata*) are two common tick-borne diseases (TBDs) of cattle in Egypt (El-Alfy *et al.*, 2022). *A. marginale*, a bacterium belonging to the order *Rickettsiales* of the family *Anaplasmataceae*, is an obligate intraerythrocytic agent (Kocan *et al.*, 2010), while *T. annulata* is a member of apicomplexan–hemoprotozoan intracellular parasites (Silva, Marques, and Oliva, 2010). In some regions, *Rhipicephalus microplus* ticks are the primary vectors for the biological transmission of *A. marginale*. However, other tick species have also been incriminated in the spread of infection in other regions. Other iatrogenic methods and transplacental transmission of *A. marginale* could also occur (Aubry and Geale, 2011; Henker *et al.*, 2020; Pothmann *et al.*, 2016). However, *T. annulata* is transmitted transstadially by *Hyalomma* spp. ticks (Gharbi *et al.*, 2020), while *H. excavatum* is Egypt's most common tick species infected with T. annulata (Al-Hosary *et al.*, 2021). Co-infection with *A. marginale* and *T. annulata* in cattle was previously reported (El-Ashker *et al.*, 2015; Hailemariam *et al.*, 2017). Furthermore, *A. marginale* was more associated with *T. annulata* co-infection than other TBPs infecting Egyptian cattle (Abdullah *et al.*, 2021; Al-Hosary *et al.*, 2020).

Anaplasmosis, often known as gall sickness, commonly infects cattle but could infect other ruminants (Aubry and Geale, 2011). It is characterized mainly by pyrexia, anemia, weakness, constipation, jaundice, loss of appetite, dehydration, depression, difficulty breathing, abortion in pregnant cows, and death (Kieser, Eriks, and Palmer, 1990). Typically, anemia and icterus are present without hemoglobinemia and hemoglobinuria because the condition causes extravascular erthrophagocytosis instead of intravascular hemolysis (Rymaszewska and Grenda, 2008). In severe cases, urine is usually colored dark brown because of the high concentration of bile pigments (Nazifi *et al.*, 2008). Severe hemolytic anemia, icterus splenomegaly, hepatomegaly, and petechial hemorrhage on the serosa surface over the heart and pericardium are the main postmortem findings (Richey, 1991). In Egypt, *A. marginale* infection in cattle, buffaloes, and camels was detected in earlier investigations (Al-Hosary *et al.*, 2020; El-Ashker *et al.*, 2015; El-Dakhly *et al.*, 2020; El-Naga and Barghash, 2016; Fereig

et al., 2017; Nasreldin *et al.*, 2020; Parvizi *et al.*, 2020; Selim *et al.*, 2021a). Additionally, DNA fragments of *A. marginale* have been detected in *H. anatolicum* and *Rhipicephalus annulatus* collected from cattle (Loftis *et al.*, 2006).

Bovine theileriosis, also known as tropical theileriosis is a chronic debilitating disease of large ruminants (Bishop *et al.*, 2004; Mans, Pienaar, and Latif, 2015), where its tick vector is inhabitant (Aktas, Altay, and Dumanli, 2006). The parasite's harmful effects on the host's immune system and lymphatic tissues determine the clinical characteristics of tropical theileriosis (El-Deeb and Younis, 2009; Vikrant Sudan *et al.*, 2012). Fever, anorexia, diarrhea, enlargement of the pre-scapular and pre-femoral lymph nodes, respiratory distress, jaundice or anemic mucous membrane, and corneal opacity of the eye are the most prominent clinical signs of theileriosis in cattle (Agina *et al.*, 2020; El-Dakhly *et al.*, 2018). Previously, some studies investigated the presence of *T. annulata* among animals in Egypt by various serological and molecular methods (El Damaty *et al.*, 2022; El-Dakhly *et al.*, 2018; Nayel *et al.*, 2012; Rizk *et al.*, 2017; Selim and Khater, 2022; Selim, Weir, and Khater, 2022; Yousef *et al.*, 2020).

This study aimed to investigate the prevalence of *A. marginale, T. annulata,* and their co-infection among cattle from six Egyptian provinces located in northern, middle, and southern regions using the molecular method. Additionally, we aimed to analyze the potential risk factors associated with *A. marginale,* and *T. annulata* infection in Egyptian cattle. We believe the current study could shed light on the silent infection of *A. marginale* and *T. annulata* in cattle in these six Egyptian provinces, which is crucial for designing and implementing effective host-specific control measures. Moreover, this study will pave the way for the researchers to conduct more efficient studies on both pathogens in the investigated regions, where the silent infections with several TBPs are poorly investigated.

2. Materials and methods

2.1. Ethical approval

The Animal Ethics Committee of the Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt, set rules for handling all animals (Approval number, DMU/VetINF-2019-/05).

2.2. Animal population and farms

This study was conducted as a part of an inclusive research project screening silent infections among animals between January 2019 and December 2022. A total of 278 apparent healthy cattle (85 beef and 193 dairies) from 24 cattle farms and four abattoirs located in six Egyptian provinces, scattered across Northern Egypt's Beheira and Damietta, Central Egypt's Cairo and Fayoum, and Southern Egypt's Qena and Luxor were investigated during 2022 (**Figure 1**; **Table 1**). These six provinces were selected for investigation based on the availability of sampling permission. Moreover, they represented the various geographical and environmental features of Egypt. The samples from cattle in each farm and abattoir (approximately 10%) were randomly collected for investigation, while the total samples from each province were based on the owner's consent for sampling. These cattle were classified into wide varieties including different ages, < 2 years (n = 24), 2-5 years (n = 111), and > 5 years (n = 143); sexes (103 males and 175 females); and breeds such as the Egyptian Native breed (n = 20), the Holstein breed (n = 68), and the Mixed breed (n = 190), which was a crossbreed between cattle from foreign breeds and Native cattle. Regarding farm size, a number of 128 Cattle blood samples were obtained from 21 small-sized farms (< 200 heads per farm), 65 blood samples were collected from cattle in three large-sized farms (> 200 heads per farm), and 85 blood samples were randomly collected from sporadic cases presented to slaughter in four abattoirs. Notably, no vaccines for bovine anaplasmosis and theileriosis are available in Egypt.

2.3. Blood sampling and DNA extraction

Using the tail vein puncture, blood samples were collected into a glass tube with an anticoagulant. The Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA) was used to extract genomic DNA from 300 µL of whole blood by the manufacturer's instructions. Using the NanoDrop One Spectrophotometer, the concentration of DNA samples was determined (Thermo Fisher Scientific; Waltham, MA, USA). For Polymerase Chain Reactions (PCRs), DNA samples were diluted in water free of nuclease to a final concentration of 100 ng/µL.

2.4. Detection of A. marginale and T. annulata using Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to identify the presence of *A. marginale msp4* and *T. annulata tams-1 genes* in the extracted DNA samples by using specific primers to amplify 344-bp and 768-bp fragments, respectively (**Table 2**) as previously described (Mossaad *et al.*, 2021). The reaction was performed in 96 well plates 0.1 mL (Applied Biosystems, Foster City, CA, USA) on a BAX Q7 thermal cycler (BAX Q7 systems, Marsiling, Singapore) using an ABT 2X qPCR SYBR Green Mix (Applied Biotechnology, China) according to the manufacture instructions. Positive control DNA samples for *A. marginale* and *T. annulata* were provided by the Department of Animal Medicine, Damanhour University, Egypt, while 1 µL of nuclease-free water was used as a negative control. Thermal cycling conditions were performed as previously described (Selim *et al.*, 2021b). The cycle threshold (Ct) values for each sample, positive and negative controls were reported. All amplified PCR products were then visualized under the ultraviolet (UV) light after electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

2.5. Statistical analysis

The prevalence of *A. marginale* and *T. annulata* in the examined animals was calculated. GraphPad Prism version 7 (GraphPad Software, San Diego, California, USA) and online statistics software http://vassarstats.net/ were used to perform the statistical analysis.

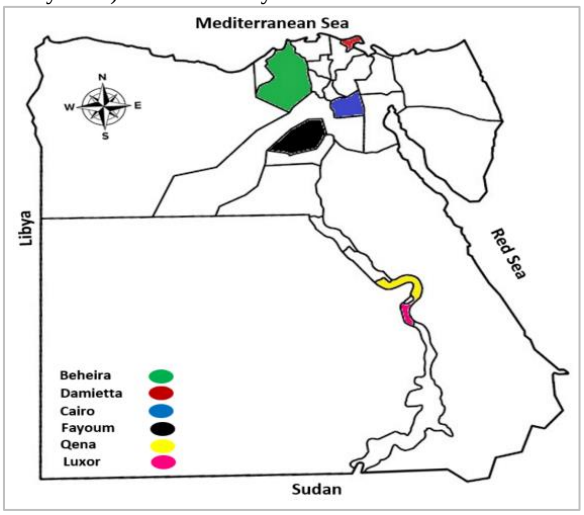

Figure 1. The geographical map of Egypt shows the location of the six provinces, where the samples were collected in this study.

Table 1. Numbers and characteristics of tested cattle from different provinces of Egypt

Province / Population	Beheira	Damietta	Cairo	Fayoum	Qena	Luxor	Total
Location in Egypt	Northern	Northern	Middle	Middle	Southern	Southern	Various
No. of tested farms : abattoirs	15 : 0	1 : 0	0 : 1	1:1	6 : 2	1 : 0	24 : 4
Farm size (Small : Large)*	14 : 1	0 : 1	0	1 : 0	5 :1	1 : 0	21 : 3
Age (years)	< 2 / 2-5 / > 5	> 5	< 2 / 2-5	2-5 / > 5	< 2 / 2-5 / > 5	< 2 / 2-5	3
Sex	Male & Female	Female	Male	Male & Female	Male & Female	Male & Female	2
Breed	Mixed / Holstein	Holstein	Mixed	Mixed / Holstein	Native / Mixed / Holstein	Native / Mixed	3
No. of samples	109	31	32	15	75	16	278

* Small-size farms (< 200 heads per farm), while large-size farms (> 200 heads per farm)

Table 2. Sequences of PCR primers used in this study

Pathogen Target Gene	Direction	Oligonucleotide Sequences (5 →3)	Annealing Temperature	Product size (bp)	References
A. marginale msp4	Forward	CTGAAGGGGGAGTAATGGG	60 ◦C	344	(Mossaad *et al.*, 2021)
	Reverse	GGTAATAGCTGCCAGAGATTCC			
T. annulata tams-1	Forward	ATGCTGCAAATGAGGAT	56 ◦C	768	(Mossaad *et al.*, 2021)
	Reverse	GGACTGATGAGAAGACGATGAG			

3. Results

The prevalence of *A. marginale* and *T. annulata* was detected in 278 DNA samples extracted from cattle blood from 24 farms and four abattoirs in six provinces representing the various Egyptian geographic and climatic regions. The total prevalence of *A. marginale* was 39.6% (110 / 278), while *T. annulata* was 5.8% (16 / 278), and the co-infection with both pathogens was 2.5% (7 / 278) (**Table 3**) as determined by RT-PCR and confirmed by the visualization of the target amplicons by UV light after gel electrophoresis. The cycle threshold (Ct) values of all positive samples were less than 27 and 26.9 cycles compared to 16.6 and 20.6 cycles in the case of positive controls of *A. marginale*, and *T. annulata*, respectively. Additionally, the Ct values of all negative samples were more than 30.5 and 36.4 cycles compared to 28.9 and 33.5 cycles in the case of negative controls of *A. marginale* and *T. annulata*, respectively. However, none of the tested samples showed Ct values between 27 and 30.5 in the case of *A. marginale* and between 26.9 and 36.4 for *T. annulata*. The target amplicons of *A. marginale* and *T. annulata* were observed at a size of 344-bp and 768-bp, respectively (**Figure 2**).

The prevalence of *A. marginale* and *T. annulata* among the cattle tested in the six provinces were incredibly variable with a prevalence of 65.1%, 0.0%, 12.5%, 13.3%, 38.7%, and 25.0% for *A. marginale* whereas 7.3%, 0.0%, 3.1%, 6.7%, 2.7%, and 25.0% for *T. annulata* in Beheira, Damietta, Cairo, Fayoum, Qena, and Luxor, respectively. Additionally, the co-infection was reported in cattle from Beheira, Qena, and Luxor, with a prevalence of 3.7%, 1.3%, and 12.5%, respectively (**Table 4**).

The influences of some risk factors such as location, age, sex, breed, production type, and farming system of the cattle tested on the prevalence of *A. marginale* and *T. annulata* were statistically analyzed. The findings indicated that the location, sex, breed, production type, and farm size were potential risks for *A. marginale* infection. In contrast, only the location and age were found to be potential risks for *T. annulata* infection. Regarding risk factors of *A. marginale* (**Table 5**), cattle from Cairo, the capital city of Egypt reported a prevalence of 12.5% (4 / 32), which was taken as a reference value, and thus cattle from Beheira (71/ 109; 65.1%; OR = 13.1; p = < 0.0001), and Qena (29 / 75; 38.7%; OR = 4.4; p = 0.01) showed a significantly higher prevalence in comparison, followed by a higher prevalence in cattle from Fayoum (2/ 15; 13.3%; OR = 1.1; p = 0.6) and Luxor (4 / 16; 25.0%, OR = 2.3; p = 0.4) but not significant. However, no positive samples were reported in cattle from Damietta (0 / 31; 0.0%). Considering age, cattle < 2 years old showed a prevalence of 37.5% (9 / 24), and thus a non-significant decrease (28 / 111, 25.2%; OR = 0.6; p = 0.31) was reported in cattle aged 2-5 years, while cattle aged > 5 years showed an increase in positivity but not significant (73 / 143, 51.0%; OR = 1.7; p = 0.27) in comparison also. For sex, female cattle showed a significantly higher prevalence (87 / 175, 49.7%; OR = 3.4; p = < 0.0001) than males (23 / 103, 22.3%). The lowest prevalence of *A. marginale* was identified in the Native cattle breed (4 / 20, 20.0%), whereas a significantly higher prevalence was shown in Mixed cattle (90 / 190, 47.4%; OR = 3.6; p = 0.03) in comparison. Moreover, the Holstein breed showed an increase in the prevalence (16 / 68, 23.5%; OR = 1.2; p = > 0.9), but not significant. Regarding the type of production, dairy cows reported a significantly higher prevalence (93 / 193, 48.2%; OR = 3.7; p = < 0.0001) than beef (17/ 85, 20.0%). Additionally, animals kept in large-size farms showed a prevalence of 24.6% (16 / 65), while, a significant increase was reported in cattle from small-size farms (77 / 128, 60.2%; OR = 4.6; p = < 0.0001) in comparison, whereas a non-significant decrease was reported in sporadic cases of abattoirs (17 / 85, 20.0%; OR = 0.8; p = 0.6).

The risk factors related to the prevalence of *T. annulata* among the cattle tested were identified (**Table 6**). The prevalence of *T. annulata* in cattle from Cairo was 3.1% (1 / 32), and thus, a significantly higher prevalence was shown in cattle from Luxor (4 / 16; 25.0%; OR = 10.3; p = 0.03) in comparison. Contrary, a non-significant difference in prevalence was reported in cattle

from Beheira (8/ 109; 7.3%; OR = 2.5; p = 0.5), Fayoum (1/ 15; 6.7%; OR = 2.2; p = > 0.9), and Qena (2 / 75; 2.7%; OR = 0.8; p = 0.7). However, no positive samples were reported in cattle from Damietta (0 / 31; 0.0%). Considering age, the highest significant prevalence of *T. annulata* was shown in cattle < 2 years old (4 / 24; 16.7%; OR = 4.6; p = 0.038), followed by cattle aged 2-5 years (6 / 111, 5.4%; OR = 1.3; p = 0. 8) but not significant against cattle aged > 5 years (6 / 143, 4.2%). For sex, males showed a prevalence of 6.8% (7/ 103), while females reported a non-significant decrease in positivity (9 / 175, 5.1%; OR = 0.7; p = 0.6) in comparison. However, the highest prevalence of *T. annulata* was shown in the Native cattle breed (2 / 20, 10.0%), while in comparison, a non-significant decrease was established in the Mixed (12 / 190, 6.3%; OR = 0.6; p = 0.6) and Holstein (2 / 68, 2.9%; OR = 0.3; p = 0.2) breeds. For the type of production, dairy cows showed a higher prevalence (12 / 193, 6.2%; OR = 1.3; p = 0.8) than beef (4/ 85, 4.7%) but were not significant. Additionally, cattle reared in large-size farms showed the lowest prevalence of *T. annulata* (2 / 65; 3.1%), while an increase in positivity was shown in comparison in cattle from small-size farms (10 / 128, 7.8%; OR = 2.7; p = 0.3), and in sporadic cases from abattoirs (4 / 85, 4.7%; OR = 1.6; p = 0.7) but not significant.

Table 3. The total prevalence of *A. marginale*, *T. annulata*, and co-infection.

Type of infection	No. of tested	No. of negative (%)	No. of positive (%)	95% CI*
A. marginale	278	168 (60.4)	110 (39.6)	33.8 – 45.6
T. annulata	278	262 (94.2)	16 (5.8)	3.4 – 9.4
Co-infection	278	271 (97.5)	7 (2.5)	1.1 – 5.3

* 95% CI calculated according to the method described by (http://vassarstats.net/). Access date January 1-3, 2023

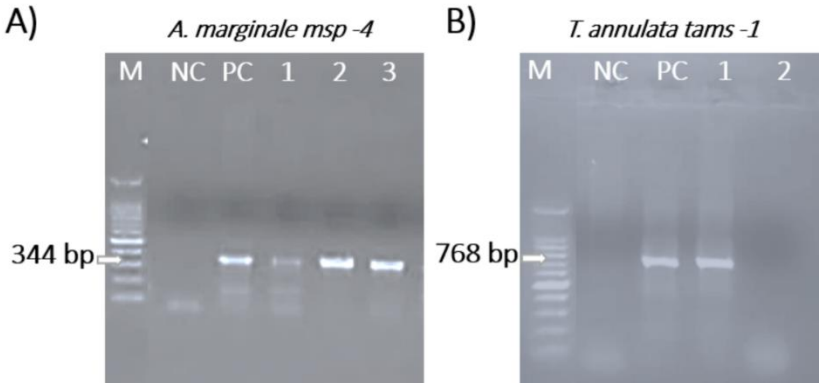

Figure 2. Ultraviolet light visualization of the results of amplification of *A. marginale msp-4* and *T. annulata tams-1* genes in DNA samples by the qRT-PCR. The amplicons were visualized in a 1.5 % agarose gel stained with ethidium bromide after gel electrophoresis for 1 hour. **(A)** Agarose gel shows *A. marginale msp-4* gene amplification results. Lane M is a molecular weight marker of 100 bp, NC is a negative control, PC is a positive control, and lanes 1 – 3 were positive tested samples. The amplicon molecular weight is 344 bp. **(B)** Agarose gel shows *T. annulata tams-1* gene amplification results. Lane M is a molecular weight marker of 100 bp, NC is a negative control, PC is a positive control, lane 1 is a positive sample and 2 is a negative sample. The amplicon molecular weight is 768 bp.

Table 4. Prevalence of *A. marginale*, *T. annulata*, and co-infection among tested cattle from different provinces of Egypt

Province	No. of tested	*A. marginale*		*T. annulata*		Co-infection	
		No. of positive (%)	95% CI*	No. of positive (%)	95% CI*	No. of positive (%)	95% CI*
Beheira	109	71 (65.1)	55.3 – 73.8	8 (7.3)	3.5 – 14.4	4 (3.7)	1.2 – 9.7
Damietta	31	0 (0.0)	0.0 – 13.7	0 (0.0)	0.0 – 13.7	0 (0.0)	0.0 – 13.7
Cairo	32	4 (12.5)	4.1 – 29.9	1 (3.1)	0.2 – 18.1	0 (0.0)	0.0 – 13.3
Fayoum	15	2 (13.3)	23.4 – 41.6	1 (6.7)	0.3 – 33.9	0 (0.0)	0.0 – 25.3
Qena	75	29 (38.7)	27.9 – 50.6	2 (2.7)	0.5 – 10.2	1 (1.3)	0.0 – 8.2
Luxor	16	4 (25.0)	8.3 – 52.6	4 (25.0)	8.3 – 52.6	2 (12.5)	2.2 – 39.6

* 95% CI calculated according to the method described by (http://vassarstats.net/). Access date January 5-7, 2023

Table 5. Risk factors analysis for the prevalence of *A. marginale* in cattle of Egypt

Analyzed factor	No. of tested	No. of negative (%)	No. of positive (%)	OR	95% CI#	*P*-value*
Location						
Beheira	109	38 (34.9)	71 (65.1)	13.1	4.3 - 36.1	< 0.0001
Damietta	31	31 (100.0)	0 (0.0)	0	0	0.1
Cairo	32	28 (87.5)	4 (12.5)	Ref	Ref	Ref
Fayoum	15	13 (86.7)	2 (13.3)	1.1	0.2 – 6.6	0.6
Qena	75	46 (61.3)	29 (38.7)	4.4	1.4 – 13.9	0.01

Analyzed factor	No. of tested	No. of negative (%)	No. of positive (%)	OR	95% CI[#]	*P*-value[*]
Luxor	16	12 (75.0)	4 (25.0)	2.3	0.5 – 10.9	0.4
Age						
< 2 years	24	15 (62.5)	9 (37.5)	Ref	Ref	Ref
2-5 years	111	83 (74.8)	28 (25.2)	0.6	0.2 – 1.4	0.31
> 5 years	143	70 (49.0)	73 (51.0)	1.7	0.7 – 4.2	0.27
Sex						
Male	103	80 (77.7)	23 (22.3)	Ref	Ref	Ref
Female	175	88 (50.3)	87 (49.7)	3.4	1.9 – 5.9	<0.0001
Breed						
Native	20	16 (80.0)	4 (20.0)	Ref	Ref	Ref
Mixed	190	100 (52.6)	90 (47.4)	3.6	1.2 – 11.2	0.03
Holstein	68	52 (76.5)	16 (23.5)	1.2	0.4 – 4.2	> 0.9
Production						
Beef	85	68 (80.0)	17 (20.0)	Ref	Ref	Ref
Dairy	193	100 (51.8)	93 (48.2)	3.7	2.0 – 6.8	<0.0001
Farm size						
Large size (> 200 head)	65	49 (75.4)	16 (24.6)	Ref	Ref	Ref
Small size (< 200 head)	128	51 (39.8)	77 (60.2)	4.6	2.4 – 9.0	<0.0001
Sporadic cases	85	68 (80.0)	17 (20.0)	0.8	0.4 – 1.6	0.6

[#] Odds ratio at 95% confidence interval as calculated by http://vassarstats.net/ (access time, January 5-7, 2023). [*] *p* value was evaluated by a Chi-square test. Ref: value used as a reference.

Table 6. Risk factors analysis for the prevalence of *T. annulata* in cattle of Egypt

Analyzed factor	No. of tested	No. of negative (%)	No. of positive (%)	OR	95% CI[#]	*P*-value[*]
Location						
Beheira	109	101 (92.7)	8 (7.3)	2.5	0.3 – 20.4	0.5
Damietta	31	31 (100.0)	0 (0.0)	0	0	1.0
Cairo	32	31 (96.9)	1 (3.1)	Ref	Ref	Ref
Fayoum	15	14 (93.3)	1 (6.7)	2.2	0.1 – 38.0	> 0.9
Qena	75	73 (97.3)	2 (2.7)	0.8	0.1 – 9.7	0.7
Luxor	16	12 (75.0)	4 (25.0)	10.3	1.0 – 102.1	0.03
Age						
< 2 years	24	20 (83.3)	4 (16.7)	4.6	1.3 – 15.9	0.038
2-5 years	111	105 (94.6)	6 (5.4)	1.3	0.4 – 4.2	0.8
> 5 years	143	137 (95.8)	6 (4.2)	Ref	Ref	Ref
Sex						
Male	103	96 (93.2)	7 (6.8)	Ref	Ref	Ref
Female	175	166 (94.9)	9 (5.1)	0.7	0.3 – 2.1	0.6
Breed						
Native	20	18 (90.0)	2 (10.0)	Ref	Ref	Ref
Mixed	190	178 (93.7)	12 (6.3)	0.6	0.1 – 2.9	0.6
Holstein	68	66 (97.1)	2 (2.9)	0.3	0.04 – 2.1	0.2
Production						
Beef	85	81 (95.3)	4 (4.7)	Ref	Ref	Ref
Dairy	193	181 (93.8)	12 (6.2)	1.3	0.4 – 4.3	0.8
Farm size						
Large size (> 200 head)	65	63 (96.9)	2 (3.1)	Ref	Ref	Ref
Small size (< 200 head)	128	118 (92.2)	10 (7.8)	2.7	0.6 – 12.6	0.3
Sporadic cases	85	81 (95.3)	4 (4.7)	1.6	0.3 – 8.8	0.7

[#] Odds ratio at 95% confidence interval as calculated by http://vassarstats.net/ (access time, January 7-9, 2023). [*] *p* value was evaluated by a Chi-square test. Ref: value used as a reference.

4. Discussion

The current study focused on the molecular detection of *A. marginale* and *T. annulata* as two major TBPs that cause negative effects on the cattle industry worldwide. Firstly, we estimated the prevalence of *A. marginale*, *T. annulata*, and co-infection in DNA samples obtained from the blood of apparently healthy cattle distributed in different geographical locations, Northern, Middle, and Southern areas of Egypt. Secondly, we analyzed the potential

risk factors associated with the prevalence of both pathogens among the cattle tested. The obtained findings gave insight into the subclinical infection with *A. marginale* and *T. annulata* among Egyptian cattle and the urgent need to apply control strategies for both infections.

Here, the overall prevalence of *A. marginale* and *T. annulata* were 39.6% and 5.8%, respectively, whereas 2.5% of the cattle tested were co-infected with both pathogens as determined by the RT-PCR. Diagnosis of both infections during the acute stage relies mainly on the microscopic examination of Giemsa-stained thin blood smears, which is still the most economical method (Al-Hosary *et al.*, 2020; El-Dakhly *et al.*, 2018; Yousef *et al.*, 2020). However, in the carrier status, the use of molecular diagnostic tools in the detection of *A. marginale* and *T. annulata* has increased in recent decades because of their higher sensitivity and accuracy than the conventional methods (El-Alfy *et al.*, 2022; Selim and Khater, 2022). Additionally, the amplification of *A. marginale* and T. annulata DNA fragments by PCR technique has been recommended to diagnose the infection in animals to be traded or traveled overseas (Díaz-Sánchez *et al.*, 2020). To detect *A. marginale*, we used amplifying primers for the membrane surface protein 4 *(msp4)* gene that was used in several previous studies (Mossaad *et al.*, 2021; Torina *et al.*, 2012). However, the tumor-associated macrophages 1 (*tams-1*) gene, which was widely used for the identification of *T. annulata* (El-Dakhly *et al.*, 2020; Mossaad *et al.*, 2021; Selim, Weir, and Khater, 2022; Yousef *et al.*, 2020) was targeted in this study. Correlating our data of the RT-PCR and visualization of the target amplicons on agarose gel revealed that the samples were positive if the Ct values were less than 27 in the case of *A. marginale* and 26.9 for *T. annulata*. However, previously, Abdullah and colleagues considered the sample positive if the Ct was less than 35 (Abdullah *et al.*, 2021).

Notably, despite the detection of *A. marginale* and *T. annulata* in high numbers of samples in this study, all of the cattle investigated were apparently healthy and none of them showed clinical signs of the diseases. In the case of *A. marginale*, this condition is known as a silent carrier that occurs because most cattle remain infected with *A. marginale* for life (Kocan *et al.*, 2010). Another cause might be the infective strain of *A. marginale*, as there are different strains of *A. marginale* globally, with various epidemiological behaviors, virulence, pathogenicity, adaptation to environmental conditions, and stimulation of the host's immune response (Cabezas-Cruz and de la Fuente, 2015). Furthermore, the infective dose could be a factor in the manifestation of the clinical signs as many as 70% or more of the erythrocytes possibly become infected during the acute stage and/or during the clinical signs (Aubry and Geale, 2011). Consistently to our findings, previous studies on *A. marginale* reported a prevalence of 28% (Fereig *et al.*, 2017), 10.6% (El-Dakhly *et al.*, 2020), 68.3% (Al-Hosary *et al.*, 2020), and 20% (Selim *et al.*, 2021a) in healthy Egyptian cattle. Conversely, Rady and colleagues in Qena province detected *A. marginale* DNA in 23% of cattle suffering from clinical signs of anaplasmosis (Rady *et al.*, 2023).

Similarly, long-term carriers of *T. annulata* occur because of the infection of a few of their erythrocytes with the parasite (Tavassoli *et al.*, 2011). These asymptomatic carriers could play an essential role in the cycle of infection as reservoirs for tick infection and, subsequently the spread of infection among animals (Gul, Kakakhel, and Akbar, 2015). In agreement with our findings, El Damaty and colleagues reported a 70% seroprevalence of *T. annulata* in 350 tested healthy cattle (El Damaty *et al.*, 2022). Moreover, *T. annulata* infection without clinical signs was also reported in 16.5% (Selim, Weir, and Khater, 2022) and 22% (El-Dakhly *et al.*, 2020) of tested cattle. In contrast, some studies detected *T. annulata* in clinically infected cattle (Yousef *et al.*, 2020), with a prevalence of 27.13% (El-Dakhly *et al.*, 2018).

Noticeably, this study recorded a relative increase in the prevalence of A. marginale than T. annulata among the cattle tested. The endemic status of *A. marginale* compared to *T. annulata* might be due to the existence of the insect vectors as well as the mode of transmission of both pathogens. Indeed, *A. marginale* is transmitted biologically by more than twenty tick species, and it could be transmitted mechanically through biting flies and blood-contaminated objects (Kocan *et al.*, 2010). Moreover, transplacental transmission possibly occurs in the case of *A. marginale* (Aubry and Geale, 2011; Henker *et al.*, 2020). However, *T. annulata* is transmitted by a limited number of ticks from the genus *Hyalomma* (Al-Hossary *et al.*, 2021; Gharbi *et al.*, 2020).

On the provincial level, the prevalence of *A. marginale* (0.0% - 65.1%) and *T. annulata* (0.0% - 25.0%) were greatly varied between regions. This variation is possibly due to environmental factors, particularly climatic conditions, which play a great role in the existence of tick vectors (Al-Hosary *et al.*, 2020). Regarding *A. marginale*, cattle from Beheira province reported the highest prevalence (65.1%), while no positive samples were identified in cattle from Damietta, where only a single farm was investigated. Consistently, previous studies reported a prevalence of 17% as determined by PCR in cattle of Beheira (Abdullah *et al.*, 2021), Dakahlia, 20.1% (El-Ashker *et al.*, 2015), Fayoum, 16% (El-Dakhly *et al.*, 2020), Beni-Suef, 4% (El-Dakhly *et al.*, 2020), El-Wadi El-Gadid, 12% (El-Dakhly *et al.*, 2020), and 68.3% (Al-Hosary *et al.*, 2020), Menofia, 15.2% (Tumwebaze *et al.*, 2020) and 19.6% (Selim *et al.*, 2021a), Kafr El-Sheikh, 22.1% (Selim *et al.*, 2021a), Gharbia, 17.7% (Selim *et al.*, 2021a), Assuit, 92.7% (Al-Hosary *et al.*, 2020), and Qena, 23% (Rady *et al.*, 2023). For *T. annulata*, cattle from Luxor reported the highest prevalence (25.0%). In this context, some studies reported the prevalence of *T. annulata* with percentages of 12.2% - 22.6% in cattle from Beheira, Alexandria, Kafr El Sheikh, Qalyubia, and Menofia (Selim, Weir, and Khater, 2022), 70% in Sharkia (El Damaty *et al.*, 2022), and 11.4% in El-Wadi El-Gadid (El-Dakhly *et al.*, 2018).

Our findings estimated that location, sex, breed, production type, and farm size were potential risks for *A. marginale* infection in cattle. In this study, the endemic nature of *A. marginale* in some provinces might be affected by the existence of insect vectors that are influenced by the climate and management (Al-Hossary *et al.*, 2021; Fereig *et al.*, 2017; Selim *et al.*, 2021a). The number and characteristics of the cattle tested could also be counted because of a shortage of samples from some regions. This study showed a higher prevalence of *A. marginale* in cattle < 2 years (37.5%) than in the older group 2-5 years (25.5%). This might be due to the large difference in the number of tested samples between cattle < 2 years (n = 24) and the older group (n = 111). The prevalence in females (49.7%) was higher than in males (22.3%), similar to the findings of Selim and colleagues (Selim *et al.*, 2021a) and contrasted with the findings of Rady and colleagues (Rady *et al.*, 2023). Mixed cattle showed the highest prevalence (47.4%) among the breeds tested. These data contrasted most of the previous findings that reported higher infections in foreign breeds (Rady *et al.*, 2023; Selim *et al.*, 2021a) and native cattle (Al-Hosary *et al.*, 2020). Dairy cows showed a higher prevalence (48.2%) than beef (20.0%), as dairy cows might be exposed to the infection by insect vectors for longer times, similar to the previous findings in Egyptian cattle (Al-Hosary *et al.*, 2020; Rady *et al.*, 2023; Selim *et al.*, 2021a). Here, the farming system was a major risk factor for *A. marginale* infection among cattle tested. Cattle in small-size farms were found to maintain the infection (60.2%) more than large-size farms (24.6%). In agreement with the findings of previous studies, this variation could be due to the well-developed management system and tick

control in the big farms in Egypt (Rady *et al.*, 2023; Selim *et al.*, 2021a). Despite the higher prevalence of *A. marginale* in older animals (51.0%), the age of infected animals was not a potential risk factor for infection. In contrast to our findings, some studies counted age as a risk for *A. marginale* infection (Al-Hosary *et al.*, 2020; Rady *et al.*, 2023; Selim *et al.*, 2021a). Neglecting age as a risk in our findings could be because of the high prevalence of *A. marginale* in young calves (37.5%) that are possibly infected in various ways such as horizontal and transplacental (Aubry and Geale, 2011; Henker *et al.*, 2020).

Regarding risks of *T. annulata* infection among the tested cattle, location and age were the only estimated potential factors. Limited risk variables in the current study are possibly due to the lower prevalence of *T. annulata* (5.8%) compared to the previous studies that estimated further risk factors in Egyptian cattle (El Damaty *et al.*, 2022; Selim, Weir, and Khater, 2022). Up to somewhat, the location of the cattle tested played a role in their infection with *T. annulata*. The highest prevalence (25.0%) was reported in Luxor province, Southern region. This variation might be reflected by ecology and management which influence the tick vector of *T. annulata* in some areas (Al-Hosary *et al.*, 2020; El Damaty *et al.*, 2022; El-Dakhly *et al.*, 2018; Selim, Weir, and Khater, 2022). Surprisingly, we reported a higher prevalence in young calves < 2 years old (16.7%) than in old ages > 5 years (4.2%). However, the low number of young calves tested (24 calves) might have affected the analysis. Contrary to our data, old cattle showed a higher prevalence of *T. annulata* than younger ages, as previously reported in Egypt (El Damaty *et al.*, 2022; Selim, Weir, and Khater, 2022).

This study had two limitations that should be mentioned. First, it was carried out on a few samples from some regions because of difficulties with the owner's consent for sampling. Second, the existing ticks were not collected and classified during the sample collection to analyze the risk of tick species in the transmission of *A. marginale* and *T. annulata* among cattle in Egypt. Therefore, we believe that a comprehensive nationwide study to detect *A. marginale* and *T. annulata* in various tick species could shed more light on the risk of transmission of both pathogens in Egyptian cattle.

5. Conclusion

In conclusion, this study presented a molecular survey on subclinical infection with *A. marginale* and *T. annulata* in cattle from six Egyptian provinces representing northern, middle, and southern regions. The findings indicated a higher prevalence of *A. marginale* compared to *T. annulata* among the cattle tested, whereas co-infection with both pathogens was also detected. Additionally, this study concluded that the potential risk factors for *A. marginale* infection in the cattle studied were locality, sex, breed, production type, and farm size. However, locality and age were the most critical risks for *T. annulata* infection. Therefore, a routine examination and treatment of *A. marginale* and *T. annulata* in Egyptian cattle and the control of insect vectors in cattle farms are highly recommended because no vaccines against *A. marginale* and *T. annulata* are currently approved in Egypt.

Authors contributions

Conceptualization and design; SM, NB, YB., Experiments, formal analysis, investigation; SM, MS, RH, BE, WG, YB., Resources, and shared materials; SM, RH., Writing—original draft, SM, RH., Writing—review and editing; SM, YB, RH.

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Declaration of competing interests

The authors declare that they have no competing interests.

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