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Hematological and Molecular Profiling of Some Blood Pathogens in Dog Breeding Farm in Egypt

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Abstract

Canine vector-borne diseases (CVBDs) are widespread arthropod-transmitted diseases that pose a significant threat to animal and human health. Despite their growing significance, little is known about the vector-born pathogen in Egypt. There is a substantial diagnostic challenge, especially when a dog is co-infected with more than one pathogen. Therefore, the current study's objective was to examine the prevalence of tick-borne pathogens in 49 German shepherd dogs that were clinically suspected of having vector-borne diseases from a farm in the Sharkia Governorate of Egypt. Microscopic blood smear examination (n=49) followed by quantitative polymerase chain reaction (qPCR, n=6) using species-specific primers of *Babesia* and *Mycoplasma* was used to establish the prevalence of each infecting pathogen. Most of the examined dogs recorded macrocytic hypochromic anemia with marked thrombocytopenia. The dog tick, *Rhipicephalus sanguineus* was morphologically identified. Blood smear analysis showed that *Babesia* spp. was the most prevalent pathogen detected with an overall prevalence of 44.9% (22/49), 18.44% (9/49) for *Mycoplasma* spp, and co-infection was found in 8.2% (4/49) dogs. Quantitative PCR identified *B. canis vogeli*, *B. gibsoni*, and *Mycoplasma haemocanis*. Babesiosis infection in this study was significantly reliant on sex, season, and age. This is the first microscopical and molecular identification of *M. haemocanis* in dogs in Egypt. This study provides a foundation for future avenues of research investigating prevalent vector-borne pathogens in endemic areas and offers crucial knowledge for future diagnostic efforts.

KEYWORDS
Babesia, Mycoplasma, Blood-borne pathogens, Molecular diagnostics, Egypt, Dogs.

INTRODUCTION

Dogs are prone to a variety of diseases, including parasitic, viral, and bacterial infections. Babesiosis (Tick-borne haemoprotozoal disease) is one of the most common diseases in dogs (Ogbu *et al*., 2018). Two species of canine *Babesia* are distinguished: *Babesia canis* and *Babesia gibsoni*. *Babesia canis* is classified into three subspecies: *B. canis canis* is transmitted by Dermacentor reticulatus, *B. canis vogeli* is transmitted by *Rhipicephalus sanguineus*, and *B. canis rossi* is transmitted by *Haemaphysalis leachi*. While *Rhipicephalus sanguineus* and *Haemaphysalis longicornis* are the vectors for *B. gibsoni* (Kiouani *et al*., 2020). Dogs infected with babesiosis show clinical signs such as fever, lethargy, and pale mucous membranes due to hemolytic anemia and haemoglobinuria, jaundice, and organ failure (Abdel-Rhman *et al*., 2015). *Babesia* is diagnosed by detecting intraerythrocytic piroplasms in peripheral blood smears under a microscope. However, despite variations in their genetics, pathophysiology, and vector connections, several species of *Babesia* cannot be distinguished through microscopic inspection of blood smears (Leisewitz *et al*., 2019). It has recently been determined through molecular phylogenetic analysis, specifically genotyping of the small ribosomal subunit 18S gene, that these subspecies known as *B. canis*, *B. rossi*, and *B. vogeli* are separate species (Petra *et al*., 2018).

Dogs are among the many vertebrate hosts that are infected by hemotrophic *Mycoplasma*s (*Haemoplasma*s), which are small cell wall deficient, uncultivable, and obligate epierythrocityc bacteria (Messick, 2004). *Mycoplasma haemocanis* (formerly known as *Haemobartonella canis*) infects dogs (Roblejo-Ariasa *et al*., 2022). Fleas, ticks, lice, and flies are responsible for the diffusion of *Haemoplasma* in cats, dogs, mice, pigs, and cattle (Willi *et al*., 2010) *Rhipicephalus sanguineus* serves as a reliable tick vector for *M. haemocanis* (Roblejo-Ariasa *et al*., 2022). *Haemoplasma* infection can cause acute hemolysis, which is accompanied by anorexia, lethargy, dehydration, weight loss, and unexpected death in affected animals. Severe haemolytic anemia has been described mainly in immune-compromised or splenectomized *Haemoplasma*-infected dogs. Finding *Haemoplasma* bacteria on peripheral blood smears stained with Giemsa stain using light microscopy yields a preliminary diagnosis (Willi *et al*., 2010). However, according to Raimundo *et al*. (2016), this assay has a low diagnostic sensitivity and specificity. Since they offer the best analytical sensitivity and specificity and enable the determination of *Haemoplasma* microbe counts, quantitative real-time PCR (qPCR) tests are currently being employed more and more (Willi

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et al., 2009) With numerous distinct primer pairs described, the 16S rRNA gene is the most frequent molecular marker employed in the *Haemoplasma* PCR-based tests so far (Willi *et al*., 2010). This study aimed to detect tick-borne protozoan pathogens and their hematological and molecular profile on a dog breeding farm in Egypt.

MATERIALS AND METHODS

Animals and Ethical approval

This study applied during the summer, and autumn seasons from June 2021 to November 2021, a total number of 49 dogs were examined from dog breeding farm imported dogs (German shepherds) in Sharkia Governorate, Egypt. As instructed by the Faculty of Veterinary Medicine at Cairo University, institutional and national criteria for the care and use of animals were followed. The ages of the dogs ranged between 3 to 5 years.

Clinical examination

Each dog was subjected to a complete clinical examination comprising general inspection of appearance, illness, posture, gait, skin, conformation, behavior, and physical examination for temperature, respiration, pulse, mucous membranes, and superficial lymph nodes (Hill *et al*., 2011).

Collection of blood samples

To prepare for the examination of blood film, complete blood count, haematological profiles, and DNA extraction, blood samples were taken from the cephalic vein of 49 dogs on EDTA as an anticoagulant (Ukwueze *et al*., 2022).

Preparation of Blood smears

The blood smears were applied on a clean slide, let to air dry, and then fixed for 10 minutes in absolute methyl alcohol before being stained with Giemsa. The smears were cleaned with tap water, allowed to air dry, and then light microscopically inspected using 40X and 100X lence power (OLYMPUS CX41, Japan) to look for intraerythrocytic hemoparasite stages (Zaki *et al*., 2021).

Complete blood count and hematological profiles

A Mindray BC-5000 Vet auto haematology analyzer (Mindray, Shenzhen, China) was used to perform a complete blood count (CBC) (Thongsahuan *et al*., 2020a). White blood cell (WBC), platelets, and WBC differential count were recorded and analyzed as part of the haematological profile, which included red blood cell (RBC) count, haemoglobin (Hb) concentration, hematocrit (HCT) value, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). The haematological results of the infected dogs and the results of healthy normal canines were contrasted.

Tick samples

Using forceps, fifty ticks were carefully removed from the dog bodies where they had attached to make sure the mouthparts were not lost. Alcohol was applied to the surrounding skin and the embedded living ticks to relax them and make it possible to draw them out whole. Ticks were put in tubes containing (5 parts of 70% ethyl alcohol: and 3 parts of glycerin). Ticks were identified by naked eyes and a simple microscope (El-Neshwy *et al*., 2020).

Molecular diagnosis

Extraction of DNA

The QIAamp DNA Blood Mini Kit (Qiagen, Germany, GmbH, Catalogue no. 51106) was utilized to extract DNA from samples.

PCR screening

A StepOnePlusTM apparatus was used to perform the amplifications (Applied Biosystems, Foster City, CA, USA) in the Biotechnology Unit, Animal Health Research Institute, Zagazig Branch, Egypt. Five µl of the extracted DNA were added to 15 µl of master mix (iQ™ SYBR Green Supermix; Bio-Rad, USA) as a template. Ten µl of iQ™ SYBR Green Supermix, 1µl each of forward and reverse primers, and 3 µl of deionized water made up the master mix content. The thermal profile was composed of 5 min of activation at 95°C, followed by 40 cycles at 95°C for 15

Table 1. Primers used for SYBR Green real-time PCR assay.

Target gene	Sequence (5'-3')	TA (°C)	Reference
Babesia spp. 18S rRNA	GTCTTGTAATTGGAATGATGG TAGTTTATGGTTAGGACTACG	60	Casati *et al.* (2006)
Mycoplasma 16S rRNA	ACG GGG ACC TGA ACA AGT GGT AGG CAT AAG GGG CAT GAT GAC TTG	60	Ravagnan *et al.* (2017)

Table 2. Primer sets used for molecular diagnosis of *Babesia* species using SYBR Green real-time PCR assay.

Target gene	Sequence (5'-3')	TA (°C)	Reference
B. vogeli	AGC AAT TGG AGG GCA AGT CT TGG CAA ACT CGA ACA CGC TA	60	Wahlang *et al.* (2019)
B. canis	TGCGTTGACGGTTTGACC A TGCCCCCAACCGTTCCTATTA	55	Birkenheuer *et al.* (2003)
B. rossi	GCTTGGCGGTTTGTTGC ATGCCCCCAACCGTTCCTATTA	50	Birkenheuer *et al.* (2003)
B. gibsoni	GCC TTTTTGGCGGCGTTT AT CTG CCT CGG TAG GGC CAA TAC	58	Wahlang *et al.* (2019)

sec (denaturation), specific annealing temperature for 30 s, and extension temperature of 60°C for 30 sec (Table 1). A threshold cycle (Ct) lower than 35 and a specific melting temperature (Tm) indicated a positive outcome after the SYBR Green fluorescence intensity and melting curve analyses were assessed. Negative (PCR master mix without DNA template) and positive controls (PRJNA218920 for *Babesia* and AY383241 for *Mycoplasma*) were included in each run. The thermal cycling protocol for the detection of different species of *Babesia* included an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation (94°C for 30 sec), specific annealing temperature (Table 2) for 15 sec (annealing) and extension (72°C for 20 sec). After each reaction, a dissociation curve analysis was carried out to validate the specificity of the amplification.

Statistical analysis and model procedure

Data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). A multivariate logistic regression model (PROC LOGISTIC; (SAS Institute Inc., 2012) was run with the level of significance set at $\alpha = 0.05$ to examine the effects of potential risk factors, including sex, age, and season on the detection of *Babesia* and/or *Mycoplasma*. The statistical model was incorporated as follows:

$$\log\left(\frac{\pi_{\text{Bab and/or Myc}}}{1-\pi_{\text{Bab and/or Myc}}}\right)=\beta_0+\beta_1\text{Sex}+\beta_2\text{Age}+\beta_3\text{Season}$$

Where $\pi_{\text{Bab and/or Myc}}$ is the probability of *Babesia* and/or *Mycoplasma* detection, β_0 is the intercept, and β_1Sex, β_2 Age,and β_3 Season are regression for sex effect, age, and season. Confidence intervals (95% CI) were described according to (Schwabe, 1982). Statistical significance was set at a p-value less than 0.05.

RESULTS

Clinical signs

Out of the 49 dogs that were examined, 35 displayed symptoms like fever, dehydration, icterus, weakness, anorexia, pale mucous membranes, and red urine.

Blood films examination

Of 49 dogs, *Babesia* spp. were detected in 22 (44.9%), *Mycoplasma* in 9 (18.4%), 4 dogs were co-infected with *Babesia* and *Mycoplasma* and 14 were negative. The *Babesia* species inside RBCs were pyriform in shape, pointed at one end, and round at the other (Figure 1). Also, *Mycoplasma* was a spherical-shaped organism located on the walls of the erythrocytes (Figure 2).

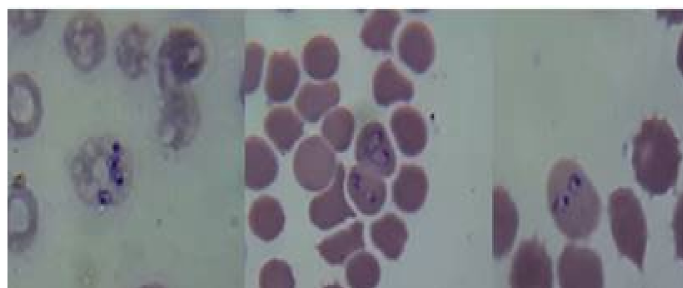

Fig. 1. Blood smear of dogs showing *Babesia* spp. in red blood cells.

Risk Factor analysis in examined dogs (sex, season, age)

The potential risk factors associated with the probability of *Babesia* and/or *Mycoplasma* detection in German dogs are demonstrated in (Table 3) The odds of *Babesia* detection were lesser in females (OR = 0.244; 75.6%; $p<0.05$) than in males. Similarly, the likelihood of *Mycoplasma* detection decreased by 38.9% (OR=0.611; $p>0.05$) in females than males. However, female dogs had 22.2% (OR=1.222; $p>0.05$) greater odds of detecting both *Babesia* and *Mycoplasma* bacteria than males. The season was another risk factor affecting *Babesia* and/or *Mycoplasma* detection. In comparison with the summer season, the probability of *Babesia* detection decreased by 79.1% (OR=0.209; $p<0.05$) during the autumn season. Also, the risk of *Mycoplasma* detection decreased by 85.4% (OR=0.146: $p<0.05$), while the aforementioned proportion become 73.5% (OR=0.265; $p<0.05$) for mixed detection of both microbes. Interestingly, the odds of *Babesia* and *Mycoplasma* detection increased steadily with increasing dog ages ($p<0.05$). Dogs at 4 and 5 years old had 2.333 and 3.022 greater odds of *Babesia* detection than their counterparts at an earlier age (3 years old). Concerning *Mycoplasma*, the risk of detection increased by 2.654 and 4.491 times at the previous age orders. Meanwhile, the likelihood of detecting both microbes increased two times in dogs at four years old ($p<0.05$).

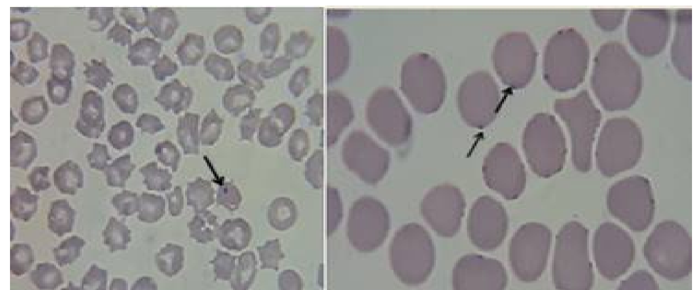

Fig. 2. Blood smear of dogs showing *Mycoplasma* attached to the wall of the red blood cells (arrows).

Morphological identification of ticks

Dogs showed ticks infestation with varying degrees on their bodies (Fig. 3). Ticks were noticed on the head (ear mostly), axilla, and trunk. *Rhipicephalus sanguineus* was morphologically distinguished groups of ticks.

Fig. 3. Ticks infestation with varying degrees on ears of dogs.

Hematological findings

The hematological profile of infected and healthy dogs was obtainable in (Table 4) as, dogs infected with *Babesia* spp. demonstrated a significant decrease ($p<0.05$) in RBCs count, Hb concentration, and PCV % compared to healthy ones while red cell indices showed a considerable increase in MCV and MCH with a significant decrease in MCHC giving the picture of macrocytic hypochromic anemia. Moreover, marked thrombocytopenia was detected (significantly decreased platelet count). Differential leukocytic count revealed a significant reduction ($p<0.05$) in total leukocytic count (leukopenia), neutrophil, and eosinophil counts. However, lymphocytic and monocytic counts were significantly

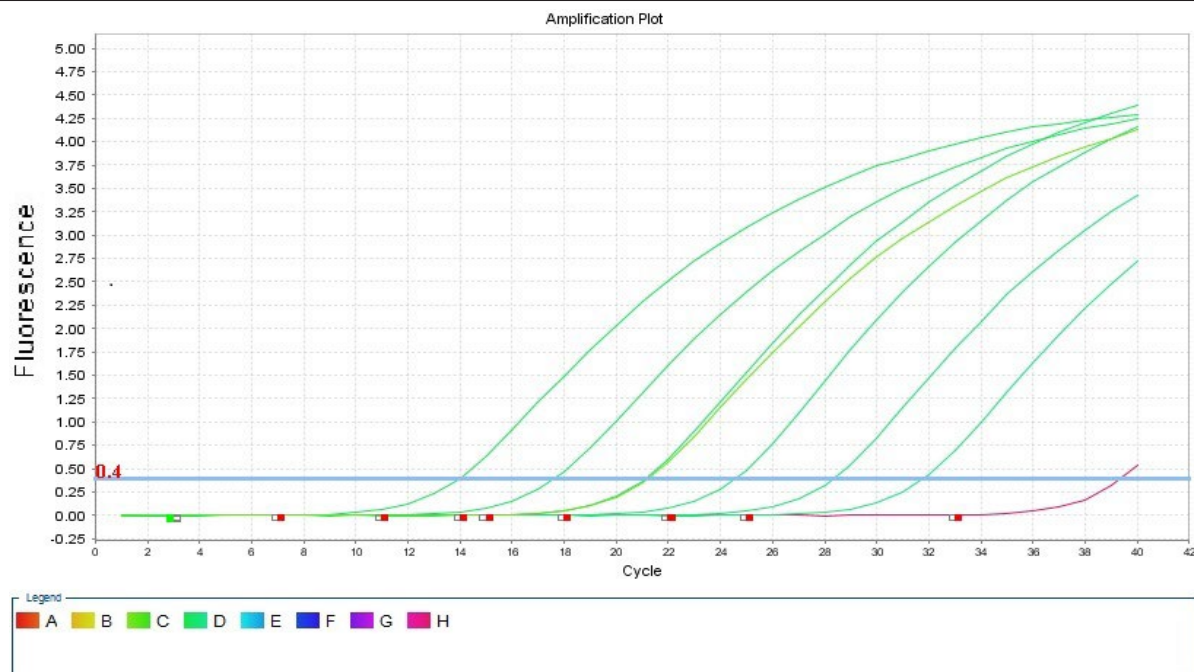

Fig. 4. SYBR Green real-time PCR amplification plot for positive results for 18S rRNA of *Babesia* in 6 samples. The upper curve represents control positive and the lower linear one represents control negative. All rest curves are parallel to positive control.

Table 3. Logistic regression analysis of different risk factors associated with the probability of *Babesia* and/or *Mycoplasma* detection in dogs.

Item[1]	*Babesia*			*Mycoplasma*			Mixed		
	B	OR	95% CI	B	OR	95% CI	β	OR	95% CI
Sex									
Male		Ref.			Ref.			Ref.	
Female	-1.41	0.244*	0.122 - 0.408	-0.49	0.61	0.284- 1.635	0.20	1.22	0.865 - 5.632
Season									
Summer		Ref.			Ref.			Ref.	
Autumn	-1.56	0.209*	0.102 - 0.412	-1.92	0.146*	0.064 - 0.211	-1.33	0.265*	0.171 - 0.360
Age									
3 Years		Ref.			Ref.			Ref.	
4 Years	0.85	2.333*	1.352 - 4.631	0.92	2.654*	0.145 - 0.964	0.69	2.000*	1.237 - 3.251
5 Years	1.11	3.022*	1.098 - 6.035	1.50	4.491*	0.090 - 0.365	ND.	ND.	ND.

β: regression coefficient; OR: odds ratio; CI: confidence interval (95%); Ref.: reference; ND: non detected; and * p<0.05.

Table 4. Hematological parameters of healthy and infected dogs.

Category	Healthy Mean± SD	*Babesia* Mean± SD	*Mycoplasma* Mean± SD	Mixed Mean± SD	P-value
RBCs (x10^6/µl)	5.68±0.67	2.69±0.46	2.04±0.45	2.66±0.29	<0.001
Hb (g/dl)	14.97±1.45	9.47±0.99	6.79±0.5	8.61±0.64	<0.001
PCV (%)	41.7±2.52	29.23±3.26	23.71±2.06	27.48±4.39	<0.001
Platelets (x10^3/µl)	288.0±57.1	127.2±25.1	81.1±6.6	134.0±7.8	<0.001
MCV (fl)	73.9±5.63	109.96±11.29	118.94±15.35	102.88±5.83	<0.001
MCH (pg)	26.46±1.89	35.67±3.72	34.15±5.04	32.55±3.14	<0.001
MCHC (g/dl)	35.86±2.06	32.45±1.2	28.66±1.5	31.82±4.49	<0.001
WBCs (x10^3/µl)	9.43±0.56	7.71±0.73	18.93±0.79	7.39±0.87	<0.001
Neutrophils (x10^3/µl)	6.5±0.39	3.7±0.34	16.09±0.67	3.71±0.44	<0.001
Lymphocytes (x10^3/µl)	2.17±0.13	3.25±0.3	1.75±0.07	2.96±0.35	<0.001
Monocytes (x10^3/µl)	0.4±0.02	0.62±0.06	1.04±0.04	0.56±0.06	<0.001
Eosinophils (x10^3/µl)	0.35±0.02	0.14±0.02	0.02±0	0.17±0.02	<0.001
Basophils (x10^3/µl)	0.015±0.005	0.014±0.005	0.005±0	0.013±0.005	<0.001

P<0.05 is a statistically significant analysis done by ANOVA.

increased.

Blood pictures of dogs infected with *Mycoplasma* showed a significant decrease in each of RBC's count, Hb concentration, and PCV% compared to healthy ones while red cell indices showed a significant increase in MCV and MCH with a significant decrease in MCHC. Platelet count showed a significant reduction compared to non-infected ones.

Leukogram results revealed a significant elevate in total leukocytic count (leukocytosis), neutrophils, and monocytes with a significant decrease in lymphocytes, and eosinophils count.

The examination of blood samples of a group of dogs that have co-infection with *Babesia* spp. and *Mycoplasma* showed significantly decreased RBCs count, Hb concentration, PCV %, and MCHC value, on the other hand, MCV and MCH revealed a significant increase compared to healthy ones. Results showed a significant decrease in platelet count compared to healthy dogs. Leukogram results revealed significantly decreased ($p<0.05$) in total leukocytic (leukopenia), neutrophilic, and eosinophilic counts with a significant increase in lymphocytic and monocytic counts.

Molecular recognition of blood-borne microorganisms

For the first primers set among the six co-infected samples, molecular screening demonstrated that all samples were principally infected with *Babesia* spp. (100%) followed by *Mycoplasma haemocanis* (33.3%) as shown in Figures 4 and 5. The second SYBR green-based real-time PCR assay that was applied to distinguish the specific type of *Babesia* showed that five samples were *B. vogeli*, and one sample *B. gibsoni* (Figure 6). These RT-PCR assays demonstrated high accuracy, producing no false positive

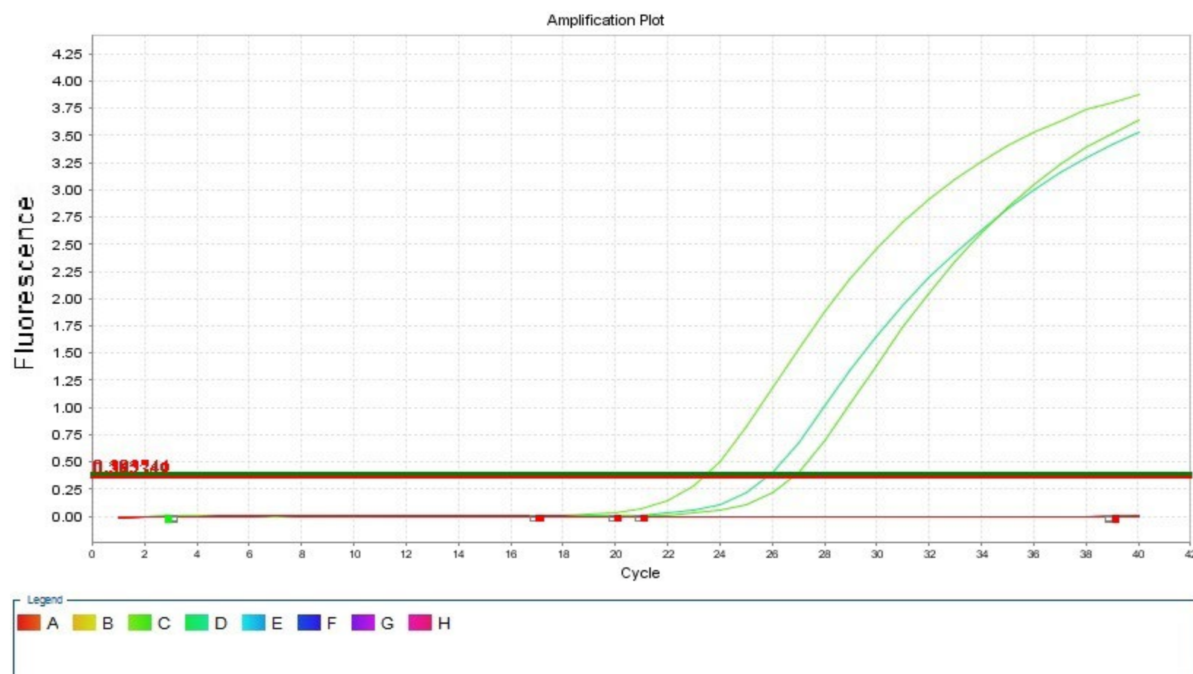

Fig. 5. SYBR Green real-time PCR amplification plot for positive results for 16S rRNA of *Mycoplasma* in 6 samples. The upper curve represents control positive and the lower linear one represents control negative. All rest curves are parallel to positive control.

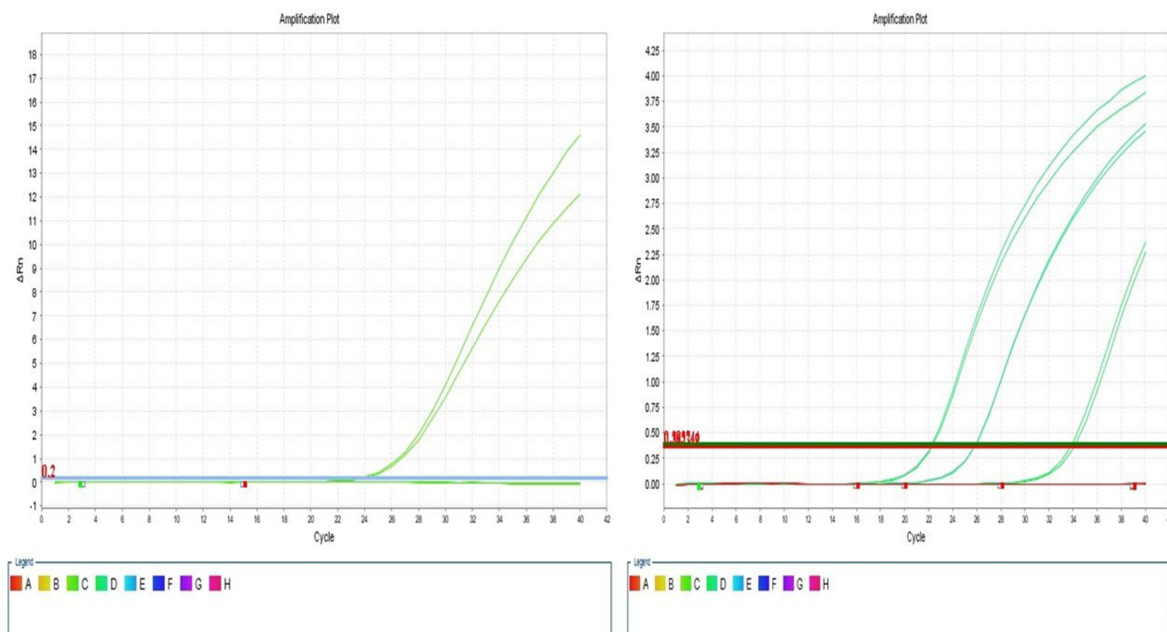

Fig. 6. SYBR Green real-time PCR amplification plots for *B. gibsoni* and *B. vogeli*. The upper curve represents control positive and the lower linear one represents control negative. All rest curves are parallel to positive control.

or false-negative results.

DISCUSSION

Clinical signs of infection with *Babesia* and *Mycoplasma haemocanis* recorded in our paper include depression, fever, tachycardia, tachypnea, lethargy, anorexia, dehydration, epistaxis, pale mucus membranes, and swollen lymph nodes; similar signs were documented (Greene, 2006; Leschnik *et al.*, 2007; Sudhakara *et al.*, 2016; Lapsina *et al.*, 2022). These clinical symptoms could be the result of tissue hypoxia following anemia and a systemic inflammatory response syndrome brought on by a detectable cytokine reduction in babesiosis (Salem and Farag, 2014).

The obtained data detected *Babesia* spp. (n.= 22) in 44.9% of Giemsa-stained smears. In Egypt, it was recorded that the rate of infection with *Babesia* spp. was 76.92%, 8.5%, and 25.62% as recorded by Salem and Farag (2014); Abdel-Rhman *et al.* (2015) and Zaki *et al.* (2021), respectively. In other countries, the prevalence rates of *Babesia* spp. in dogs were 30% in Romania (Thongsahuan *et al.*, 2020b), 9.56% in Thailand (Andersson *et al.*, 2017), and 13.22% in Algiers (Kiouani *et al.*, 2020).

The percentage of *Mycoplasma* spp. infection in the obtained finding was 18.4% which agreed with Andersson *et al.* (2017) who detected the similar prevalence rate.

Total erythrocytic count in this study was diminished in dogs with babesiosis; dogs infected with *M. haemocanis*, and co-infected dogs compared to healthy ones which gave a picture of anemia. It was mentioned that autoantibodies are directed against elements of the membranes of infected and uninfected erythrocytes, and this causes intravascular and extravascular hemolysis, which results in anemia (Sudhakara *et al.*, 2016). *Babesia* initiates a mechanism of antibody-mediated cytotoxic destruction of circulating erythrocytes. Anemia may be more reliant on the host immunological response than on the piroplasm directly destroying RBC (Boozer and Macintire, 2003). The obtained results indicated that *Babesia*, *M. haemocanis* infection, and co-infection with *Babesia* species and *M. haemocanis* cause macrocytic hypochromic regenerative anemia. The data were parallel with some of the research, which recorded normocytic and normochromic anemia in 74% of dogs with babesiosis (Furlanello *et al.*, 2005). In addition, macrocytic hypochromic regenerative anemia was reported in canine babesiosis (Davitkov *et al.*, 2015). Moreover, Ajayi *et al.* (2009) documented macrocytic hypochromic anemia in a female mongrel dog infected with *M. haemocanis*.

The obtained results revealed lower Hb, PCV, and MCHC with higher MCV volumes in dogs infected with *Babesia* compared to healthy dogs and this agreed with Niwetpathomwat *et al.* (2006) and Thongsahuan *et al.* (2020b). A significant decrease in MCHC (hypochromasia) was observed by Fleischman (2012) as a direct outcome of parasitizing *Babesia* organisms and destructive RBCs.

Thrombocytopenia, which was seen in dogs with babesiosis, was one of the obvious anomalies in the parameters that were studied in this study, which agreed with Shah *et al.* (2011); Sivajothi *et al.* (2014) and Sudhakara *et al.* (2016), and in contrast to Bilwal *et al.* (2017) who reported non-significant change in platelets count between dogs infected with *B. gibsoni* and healthy dogs.

Thrombocytopenia was also observed in dogs infected with *M. haemocanis* and this resulted following Ajayi *et al.* (2009) and Lapsina *et al.* (2022) who recorded severe thrombocytopenia with acute canine hemoplasmosis.

The mechanisms of thrombocytopenia may be caused by immune-mediated platelet destruction, platelet sequestration in the spleen, or the emergence of disseminated intravascular coagulation (Boozer and Macintire, 2003). Furthermore, the obtained results showed leucopenia and neutropenia in dogs with babesiosis similar to those reported by Bilwal *et al.* (2017) and Thongsahuan *et al.* (2020b) who found neutropenia and significant leucopenia in canine babesiosis. But this result contrasts with Shah *et al.* (2011) and Bilwal *et al.* (2017) who mentioned that neutrophils significantly increased in dogs with babe¬siosis than in healthy dogs.

Results in this study revealed lymphocytosis and monocytosis in dogs with babesiosis and these results are in harmony with Rawangchue and Sungpradit (2020) but disagree with Bilwal *et al.* (2017) who noted a non-significant change in monocytic count between dogs with babesiosis and healthy dogs. In the present study, leukocytosis was detected in dogs infected with *M. haemocanis*, and this agrees with Hoskins (1991) and in disagreement with Messick (2003).

The major goal of this research was to develop a sensitive real-time PCR for detecting *Mycoplasma* and *Babesia* infections that harm dogs. The *Babesia* genus-specific primer sets for *Babesia* rossi, *Babesia vogeli*, *Babesia canis*, and *Babesia gibsoni* were designed to validate a Syper Green real-time PCR assay.

Of interest, this study offers the molecular analysis of *B. vogeli* in dogs in almost examined samples in addition to *Babesia gibsoni*, and it will be useful for verifying clinical symptoms and investigating epidemiological risk factors in dogs.

PCR inquiry of the current investigation was recognized as more sensitive than microscopic examination. It was demonstrated that compared to PCR, which can detect *Babesia* parasites and *Mycoplasma* per µl of blood, light microscopy has a detection limit for parasitemia of about 0.001%. PCR-based techniques targeting the 18S rRNA gene can be very sensitive and specific (Matsuu *et al.*, 1982; El-Demerdash *et al.*, 2023; El-Demerdash and Raslan, 2019), While three different PCR assays were compared that targeted the 18S RNA gene to diagnose *Babesia* species (Souza *et al.*, 1982). The SYBR green assay (Bloch *et al.*, 2013) was the most sensitive (but not specific).

Various authors estimated the infection rates based on molecular genetic assay; the incidence of *B. canis* infection in Sao Luis, Brazil was 3.3% (Silva *et al.*, 2012), 3.75% in Iran (Razi *et al.*, 2013), and 3% in India (Sarma *et al.*, 2019), those are all less prevalent than the prevalence found in the current study. The environment, which favors the transmission of blood-borne infections, as well as the age and gender of the canines, probably likely have an impact on regional prevalence. Tick distribution and abundance are important epidemiological indicators because of their local and seasonal variations (Jain *et al.*, 2018).

Co-infections with vector-borne diseases may cause more severe symptoms and different clinical illness manifestations compared to isolated infections., which can make diagnosis and treatment more difficult (Tuttle *et al.*, 2003; Mylonakis *et al.*, 2004; Baxarias *et al.*, 2018). As a result, the RT-PCR test may be useful for screening investigations of canine asymptomatic infections and simultaneous species-specific detection of 18S rRNA of *B. canis* and 16S rRNA of *Mycoplasma* infections in clinical samples.

In this study, it was recorded co-infection with *Babesia vogeli* and *M. haemocanis* in 2 dogs and this finding agreed with Mascarelli *et al.* (2016) who recorded co-infection with *Babesia vogeli* and *Mycoplasma haemocanis* in 3% of examined dogs in their study.

The obtained results indicated point to a challenge that veterinarians may face when identifying positive cases, not only because of co-infections that make the diagnosis more difficult but also because they make a person more vulnerable to infections by other hemoparasites (such as Ehrlichia, Rickettsia, Anaplasma, among others) as stated by Rojas *et al.* (2014) and Happi *et al.* (2018).

Risk factors for co-infection prevalence were significantly influenced by sex, age, and season. The infection rate was high in dogs at the age of 4 years, these findings agree with earlier research by Hornok *et al.* (2006), who found that canine babesiosis increases with age, peaks between the ages of 3 and 5 years, and then declines.

The high prevalence of blood-borne infections in young dogs may be a consideration of their immature immunity and gregariousness. Compared to adult and elderly dogs, which may be less likely to roam, this is likely to enhance their odds of encountering vectors (Obeta *et al.*, 2020).

The summer months were the best for the transmission of infection because of the favorable and permissive climate that the current study region offers to tick survival and reproduction all year long especially in the August and September months (Hornok *et al*., 2006; Obeta *et al*., 2020).

Male dogs reported more infections than female dogs did. Male dog's aggression and hormonal conditions could play a role in this (Van Zyl, 1995; Hussein and Hamad, 2022). At higher testosterone levels, intact male dogs have a greater incidence of aggression than females or neutered males. Generally, testosterone has an immunosuppressive effect while estrogen has an immunoenhancing effect on the immune system. Estrogen has been shown to regulate immune response by impairing negative selection of high affinity auto-reactive B cells, modulating B cell function, and leading to Th2 response (Markle and Fish, 2014).

CONCLUSION

Molecular diagnosis should be utilized to routinely diagnose blood-borne illnesses because clinical, light microscopy or hematological findings alone cannot provide a reliable diagnosis. These findings further highlight the necessity to suggest dependable acaricidal products to clients who own dogs in Egypt to avoid tick infestations, even in the absence of infestations that may be seen.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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