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Molecular, epidemiological, and hematological evaluation in *Ehrlichia canis* infected dogs from an endemic region in Egypt

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ABSTRACT

Background: Canine monocytic ehrlichiosis (CME) is considered a multisystemic, life-threatening, rickettsial, and tick-borne disease that affects canine species and is caused by *Ehrlichia canis (E. canis)*. Clinical signs of CME vary from asymptomatic to severe illness with three clinical phases. *E. canis* has the potential to infect humans.

Aim: This study aimed to provide recent information as there is limited data about the disease in Egypt. Therefore, this work was conducted to study the molecular prevalence of *E. canis* and evaluate the corresponding risk factors, hematology, biochemistry, and molecular characterization of the genus Ehrlichia and *E. canis* species among Egyptian dogs.

Methods: One hundred eighty dogs of both sexes from 3 months to 8 years from different breeds: stray and foreign breeds were examined for clinical signs in all seasons in two delta governorates: El-Dakahlia and El-Gharbia. Blood samples were collected from dogs for microscopic and haemato-biochemical analysis, and then molecular characterization of the genus Ehrlichia and species-specific *E. canis* was performed, followed by sequencing and phylogenetic analysis.

Results: Out of 180 samples examined by polymerase chain reaction (PCR) assay, 42 (23.33%) were positive for the genus of Ehrlichia and the species-specific *E. canis.* Only twenty-four dogs (13.33%) were positive for PCR, infested with ticks, and showed fever, anemia, loss of body weight, pale mucous membrane of gum and conjunctiva, blindness, paralysis, hemoglobinuria, and Melena. The univariate logistic regression revealed that all variables, including age, season, tick infestation, hemorrhage from natural orifices, and ectoparasitic treatments per year, showed statistical significance ($p \leq 0.05$), except breed and sex, which also did not exhibit any relation between CME infection in multivariate logistic regression. The presence of morulae inside leukocytes in 66 dogs out of the total examined 180 (36.67%), only 39 (59.1%) were positive for morulae and PCR-positive for *E. canis*. Dogs positive for *E. canis* suffered from anemia, severe thrombocytopenia, the absolute value of WBCs and their fractions, alanine aminotransferas (ALT), AST, ALKP, γ-GT, total. P, T.BIL, urea, globulin, and creatinine were significantly increased in dogs infected with *E. canis* when compared to those with negative PCR results, while the levels of albumin and A: G ratios were significantly decreased.

Conclusion: The current study proves the existence of *E. canis* in El-Dakahlia and El-Gharbia governorates, and this is the first large-scale study concerning the epidemiological, clinicopathological examination, molecular characterization, sequencing, and phylogenetic analysis of reported from the center of the Delta of the Nile in Egypt.

Keywords: Dogs, Egypt, *Ehrlichia canis*, Molecular, Risk factors**.**

Introduction

Canine monocytic ehrlichiosis (CME) is considered a multisystemic, life-threatening, rickettsial, and tick-borne disease that affects canine species all over the world caused by *Ehrlichia canis (E. canis),* which is an obligate intracellular Gram-negative, (Harrus and Waner, 2011; Sainz *et al*., 2015), and transmitted by the brown tick of dogs (Rhipicephalus sanguineus) from the genus Rhipicephalus which related to family Ixodidae (Groves *et al*., 1975; Aguirre *et al*., 2004).

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Ehrlichia canis has the potential to infect humans and cause clinical signs similar to CME (Maeda *et al*., 1987; Perez *et al*., 2006).

Clinical signs of CME vary from asymptomatic to severe life-threatening illness, so there are three clinical phases of CME; Acute, chronic, and subclinical. The acute phase, which lasts 2–4 weeks and is characterized clinically by anorexia, depression, lethargy, pyrexia, loss of body weight, generalized lymphadenopathy, pale mucous membranes, and hemorrhagic tendencies with/without ocular signs like anterior uveitis (Skotarczak, 2003; Mylonakis *et al*., 2019; Piratae *et al*., 2019). Some dogs may spontaneously clinically improve without treatment but remain persistent asymptomatic carriers for months or even years, which is called the subclinical phase that is characterized by absence or mild clinical signs (Sainz *et al*., 2015). Sometimes, the acute and chronic phases may be difficult to distinguish clinically. Otherwise, the chronic stage is usually more severe (Mylonakis *et al*., 2004; Harrus and Waner, 2011).

Ehrlichia canis can be diagnosed initially by microscopic examination of blood smears for the demonstration of the morula inside leucocytes (especially monocytes), but it does not appear in all positive cases due to the low levels of parasitemia (Salib and Farghali, 2015). The presence of multiple rounded and relatively large morulae within macrophages or engulfing nuclear material and the formation of typical vacuoles are clear indications of CME (El-Dakhly *et al*., 2021).

Hematological findings are diagnostic as thrombocyte count significantly decreased. During the acute stage, there are distinctive hematological findings which are true thrombocytopenia (from 20,000 to 52,000/µl), mild anemia, and mild leukopenia (Harrus and Waner, 2011), while the chronic stage is characterized by marked pancytopenia because of the hypoplasia of the bone marrow (Aziz *et al*., 2023). Many biochemical alterations aid in the diagnosis of CME including; hyperproteinemia, hypergammaglobulinemia, hyperglobulinemia, hypoalbuminemia, a slight increase in hepatic enzymes (alanine aminotransferase (ALT), ALP), and also an increase of both creatinine and blood urea nitrogen (BUN) values (Aziz *et al*., 2023).

Polymerase chain reaction (PCR) is a highly reliable and sensitive molecular method that can detect DNA of *E. canis* (4–10 days after infection) before any other traditional methods. It could be hard to detect the disease during the acute stage by microscopical examination because of the limited number of morulae (Salem *et al*., 2014; Chua *et al*., 2020). Recently, CME has been reported in Egyptian dogs, so this study aimed to provide recent information as there is limited data about the disease in Egypt. Therefore, this work was conducted to study the molecular prevalence of *E. canis* and evaluate the corresponding risk factors, hematology, biochemistry, and molecular characterization of the genus Ehrlichia and *E. canis* species among Egyptian dogs.

Materials and Methods

Study area

Our study was conducted on two delta governorates: El-Dakahlia (31°2′25″N, 31°22′58″E) and El-Gharbia (30°58′07″N, 31°09′49″E), which are located in the center of the Delta of the Nile in Egypt (Fig. 1). These Governorates are characterized by warm temperatures most of the year (between 25:40°C), which is considered a suitable condition for the multiplication and growth of ticks which are the principal vector of CME.

Animals

This study was conducted from July 2022 to December 2023 in all seasons on 180 dogs (clinically affected dogs exhibited signs such as fever, anorexia, epistaxis, and paralysis and apparently healthy dogs) of both sexes from 3 months to 8 years from different breeds: stray and foreign breeds (Table 1). Dog's localities were dog farms, streets, the Veterinary Teaching Hospital (Mansoura University), and various veterinary clinics with the owners' consent in El-Dakahlia and El-Gharbia governorates.

Sample collection

Blood samples were collected from the cephalic or saphenous veins of 180 dogs from different breeds reared in Egypt. Data of each examined animal was recorded, such as age, sex, breed, presence of ticks, season, presence of clinical signs, pregnancy, lactation, and the number of ectoparasitic treatments per year. Each animal's blood was drawn aseptically twice: in a 2 ml VACUETTE® plain tube (Greiner Bio-One, GmbH, Austria) for serum separation by using Luxiangyi TD4 centrifuge (Shanghai, China) for further biochemical tests and in a 2 ml VACUETTE® tube containing Tri potassium-EDTA for blood smear examination, complete blood count, and molecular characterization. After collection, blood samples were stored in an icebox and finally brought to the Infectious Diseases Laboratory, Faculty of Veterinary Medicine, Mansoura University, for immediate hematology and biochemical studies, and then whole blood was kept at −20°C until molecular examination at Animal Health Research Institute of Dokki, Giza, Egypt.

Microscopic and clinicopathological examinations

Blood smears on positively charged glass slides were stained with Diff-Quik for the microscopical examination for demonstration of intracytoplasmic morulae of *E. canis* inside monocytes. Total erythrocytes, total leukocytes, and thrombocytes were counted by an automated hematology analyzer. Hb, PCV, MCV, MCH, and MCHC were estimated. Serum samples were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (Ɣ-GT), alkaline phosphatase (ALKP), total protein (Total. P), albumin, total bilirubin (T.BIL), BUN, and creatinine using AGAPPE Diagnostics LTD

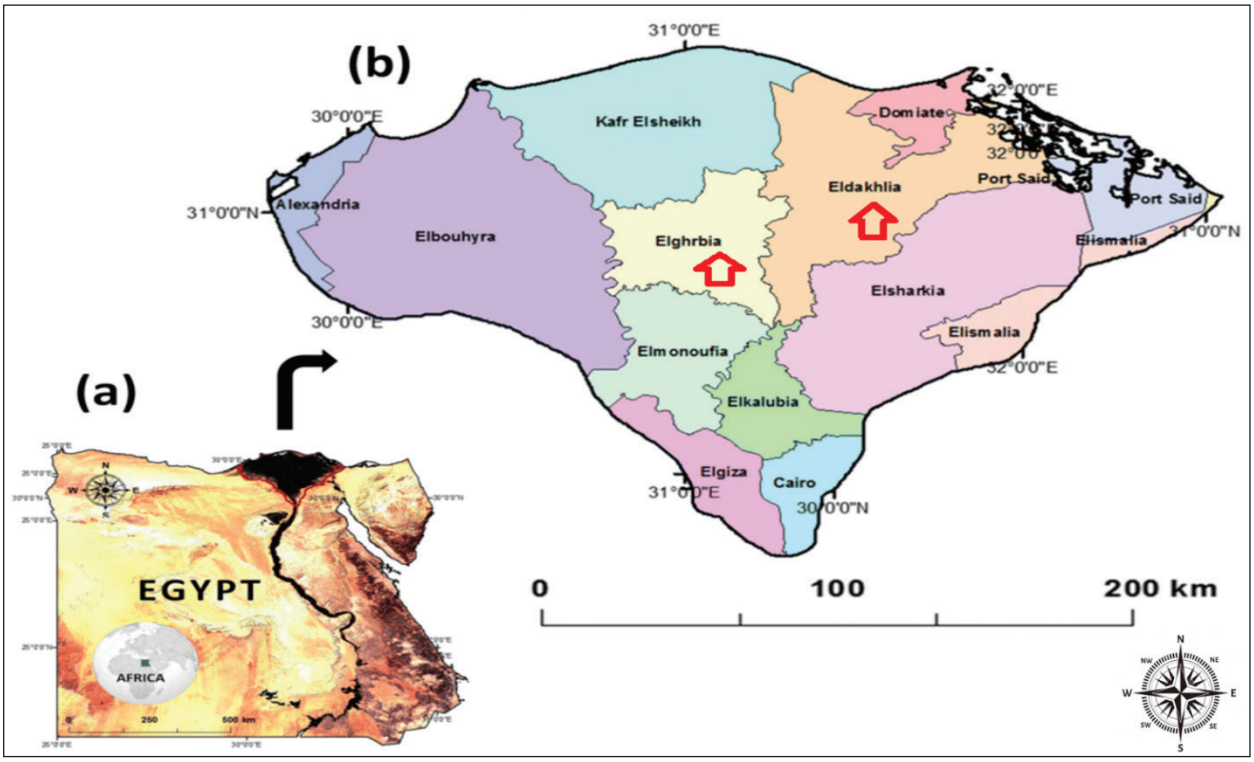

Fig. 1. Map of Egypt (a) showing El-Dakahlia and El-Gharbia governorates in the center of the Delta of the Nile in Egypt (b) where animals included in the study were sampled.

Table 1. Shows the two groups of dogs and their number.

Breeds	Stray group	Foreign breeds group					Total number
		Black German Shepherd	German Shepherd	Pit bull	Golden Retriever	Grand Danois	
Number of dogs	48			132			180
	48	24	78	12	12	6	

® kits (Ernakulam, Kerala, India) with the aid of chem-7 analyzer (Erba Mannheim, Germany) as outlined by the supplier company.

PCR and Sequencing

DNA extraction and PCR amplification

Genomic DNA was extracted from 200 µl of anti-coagulated blood from each dog using a commercially available DNA mini kit (QIAamp DNA mini kit) following the manufacturer's instructions. The purity and integrity of genomic DNA were examined using nanodrop and by running on 1% agarose gel. After quality checking, the extracted DNA was stored at −20°C until being used in the PCR assay. Conventional PCR was carried out using two pairs of primers: EHR16SD (**5'-**GGTACCYACAGAAGAAGTCC**-3'**) combined with the reverse primer EHR16SR (**5'-**TAGCACTCATCGTTTACAGC**-3'**) to amplify Ehrlichia *genus* 16SrRNA and ECA (**5'-**AACACATGCAAGTCGAACGGA**-3'**) combined with the reverse primer HE3 (**5'-**TATAGGTACCGTCATTATCTTCCCTAT**-3'**) to amplify species-specific *E. canis* 16SrRNA, the lengths of amplified products were 345 bp and 400 bp, respectively (Gal *et al*., 2008).

Preparation of PCR Master Mix for each of the tested genes according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit. The PCR amplification was done in a 25 µl reaction volume, containing 12.5 µl of Emerald Amp GT PCR mastermix (2x premix), 5.5 µl of PCR grade water, 1.0 µl of 20 pmol of each primer (forward and reverse), and 5 µl of template DNA. The DNA of an Ehrlichia genus-free dog was used as a negative control. The PCR amplifications were performed under the following cycling conditions:

initial denaturation of one cycle of 94˚C for 3 minutes, followed by two cycles of 94˚C for 30 seconds, 62˚C for 30 seconds, and 72˚C for 30 seconds. Then, two more cycles of 94˚C for 30 seconds, 60˚C for 30 seconds, and 72˚C for 30 seconds, followed by two cycles of 94˚C for 30 seconds, 58˚C for 30 seconds, 72˚C for 30 seconds, two more cycles of 94˚C for 30 seconds, 56˚C for 30 seconds 72˚C for 30 seconds, two cycles of 94˚C for 30 seconds, 54˚C for 30 s, and 72˚C for 30 seconds. Finally, thirty-nine cycles of 94˚C for 30 seconds, 52˚C for 30 seconds, and 72˚C for 30 seconds, followed by the final extension step at 72˚C for 10 minutes.

Twenty µl of each uniplex PCR product were loaded to electrophoresis on a 1% agarose gel containing ethidium bromide dye in Tris borate EDTA (TBE) buffer. The power supply was 1-5 volts/cm of the tank length for about 30 min, then the gel was transferred to a UV cabinet, photographed by a gel documentation system, and the data were analyzed through computer software.

The highly bright thick band from positive samples of *E. canis* was selected for DNA sequencing. QIAquick PCR Product extraction kit. (Qiagen Inc. Valencia CA): was used for PCR product purification and Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster City, CA) cat-number 4336817: was used for performing gene sequencing using an Applied Biosystems 3,130 genetic analyzer (HITACHI, Japan). The sequences were aligned using the programs BioEdit and MEGA6. The partial *E. canis* gene sequence was compared and analyzed to other published gene sequences in the GenBank. Furthermore, the analysis of DNA sequencing data was by nucleotide basic local alignment search tool (nBLAST) provided in the National Center for Biotechnology Information (NCBI) database to ascertain the homology of *E. canis* genes. A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 12.1 of MegAlign module of Lasergene DNAStar software Pairwise (Madison, WI). Phylogenetic analyses were done using maximum likelihood, neighbor-joining, and maximum parsimony in MEGA6 (Tamura *et al*., 2013). The sequence discovered during this work was imported into the GenBank database.

Statistical analysis

The data were analyzed with IBM SPSS Statistics® version 20 (Armonk, NY: IBM Corporation). The normal (Gaussian) distribution of obtained haemato-biochemical data were determined by performing the Shapiro-Wilk test. Data were clustered toward the mean, and *p* values exceeded 0.05, which confirms the assumption of data homogeneity. Therefore, the result outlined as mean ± standard deviation (SD) and independent samples t-test was applied to get the significance of the difference between groups. Descriptive statistics of risk factor distribution were conducted to determine the prevalence of Ehrlichiosis in dogs using the chi-square test. Logistic regression was used to assess the association between the occurrence of CME and risk factors. First, univariate logistic regression was performed where the dependent dichotomous variable was the category of the dogs, either infected positive-PCR or non-infected negative-PCR, while the suggested risk factors were the independent variables. The multivariate logistic regression was performed for the independent factors that showed a significant correlation ($p<0.1$) in univariate analysis. All statistical analysis findings were regarded as significant when $p < 0.05$ was reached. The Eta (η) coefficient test was performed to find the strength of the association between nominal independent variables (risk factors) and interval dependent variables (haemato-biochemical parameters) where ≤0.19 is no association, 0.2 to 0.39 is a weak association, 0.4 to 0.69 is a medium association, and ≥0.7 is a strong association (Field *et al*., 2009). Determined Eta values, the pattern of association between risk factors, and measured parameters were collectively represented as a Chord Diagram in OriginPro 2022 software for Windows.

Ethical approval

Samples collection in the current study was performed with the owner's permission. All procedures of the current study were carried out under the ethical standards of the Faculty of Veterinary Medicine, Mansoura University, since the samples were taken under physical restrictions and according to the ethical rules with Application No. M/159.

Results

Clinical signs

Out of 180 dogs clinically examined, 24 showed clinical signs with a prevalence of (13.33%). The affected dogs were infested with ticks and displayed various clinical signs such as fever of more than 40°C, lethargy, anemia, loss of body weight, incoordination, tachycardia, pale mucous membrane of gum and conjunctiva, shivering, blindness, paralysis, hemoglobinuria, black and tar-like feces with a jelly-like consistency (Melena), and some dogs died (Fig. 2).

Microscopic examination

Examination of Diff-Quik blood smears under an oil immersion lens showed the presence of intracytoplasmic inclusion bodies (morulae) of *E. canis* inside leukocytes (Fig. 3). The total number of blood smears examined was 180. Out of which, 66 (36.67%) dogs were positive for morulae, either infected or non-infected cases, and only 39 (59.1%) were positive for morulae and infected with *E. canis,* which was confirmed by PCR.

Clinicopathological examinations

As illustrated in Table 2, dogs positive for *E. canis* were suffered from anemia of normocytic normochromic (non-regenerative) type which outlined by the significant decrease of RBCs ($p \leq 0.001$), Hb ($p \leq 0.001$) and PCV ($p \leq 0.001$) together with the insignificant

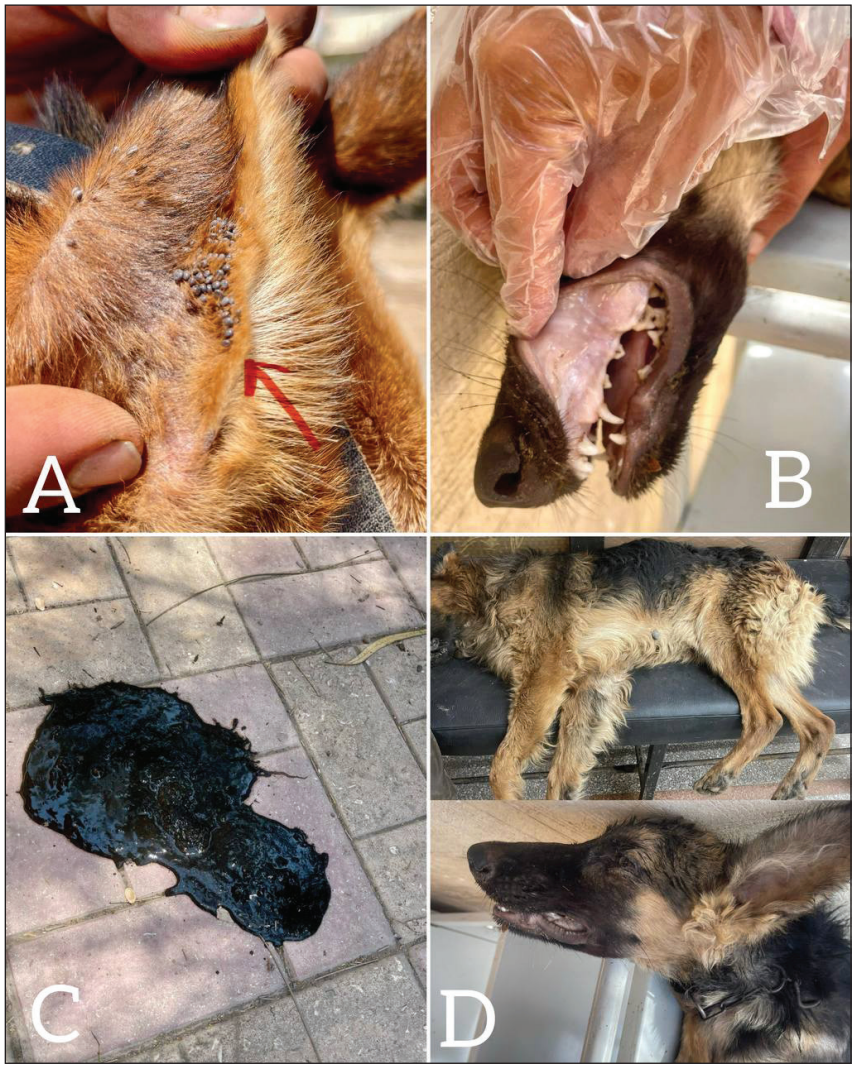

Fig. 2. Shows various symptoms of infected dogs with *E.canis*. (A) Severe infestation of ticks on dog's ear. (B) Pale gum. (C) Melena of an infected dog. (D) Lethargy.

variation of RBCs indices such as MCV (normocytic, $p = 0.93$) and MCHC (normochromic, $p = 0.47$) which were within the reference intervals. Regarding the leukogram, the absolute value of WBCs and their fractions (neutrophils, lymphocytes, monocytes, and eosinophils) were significantly increased in dogs infected with *E. canis* when compared to those with negative PCR results. Furthermore, the mean platelets count was significantly decreased ($p \leq 0.001$) in the *E. canis* infected group and reached 76.73 ± 22.60, which was below the minimum normal value of the reference interval, indicating severe thrombocytopenia. In terms of serum biochemical profile for *E. canis* infected dogs (Table 2), the values of ALT, AST, ALKP, γ-GT, total. P, T.BIL, urea, globulin, and creatinine exceeded the reference values and were significantly increased ($p \leq 0.001$) in comparison with the non-infected dogs. However, the levels of albumin and A: G ratios were significantly decreased ($p \leq 0.001$).

Epidemiology

The number and prevalence of positive cases of *E. canis* in different breeds are demonstrated in Table 3, while Table 4 displays the epidemiological data of dogs that were gathered and presented in the Table. The use of univariate logistic regression revealed that all variables showed statistical significance ($p \leq 0.05$), except breed and sex, which also did not exhibit any relation between CME infection in multivariate logistic regression, and the prevalence was higher in stray breeds (25%, $p > 0.05$, OR = 0.882, 95% CI: 0.409–1.905) than in foreign breeds and higher in males (23.8%, $p > 0.05$, OR = 1.094, 95% CI: 0.511–2.342) than females.

Meanwhile, there is a significant association between the age and occurrence of CME ($p < 0.05$), and the

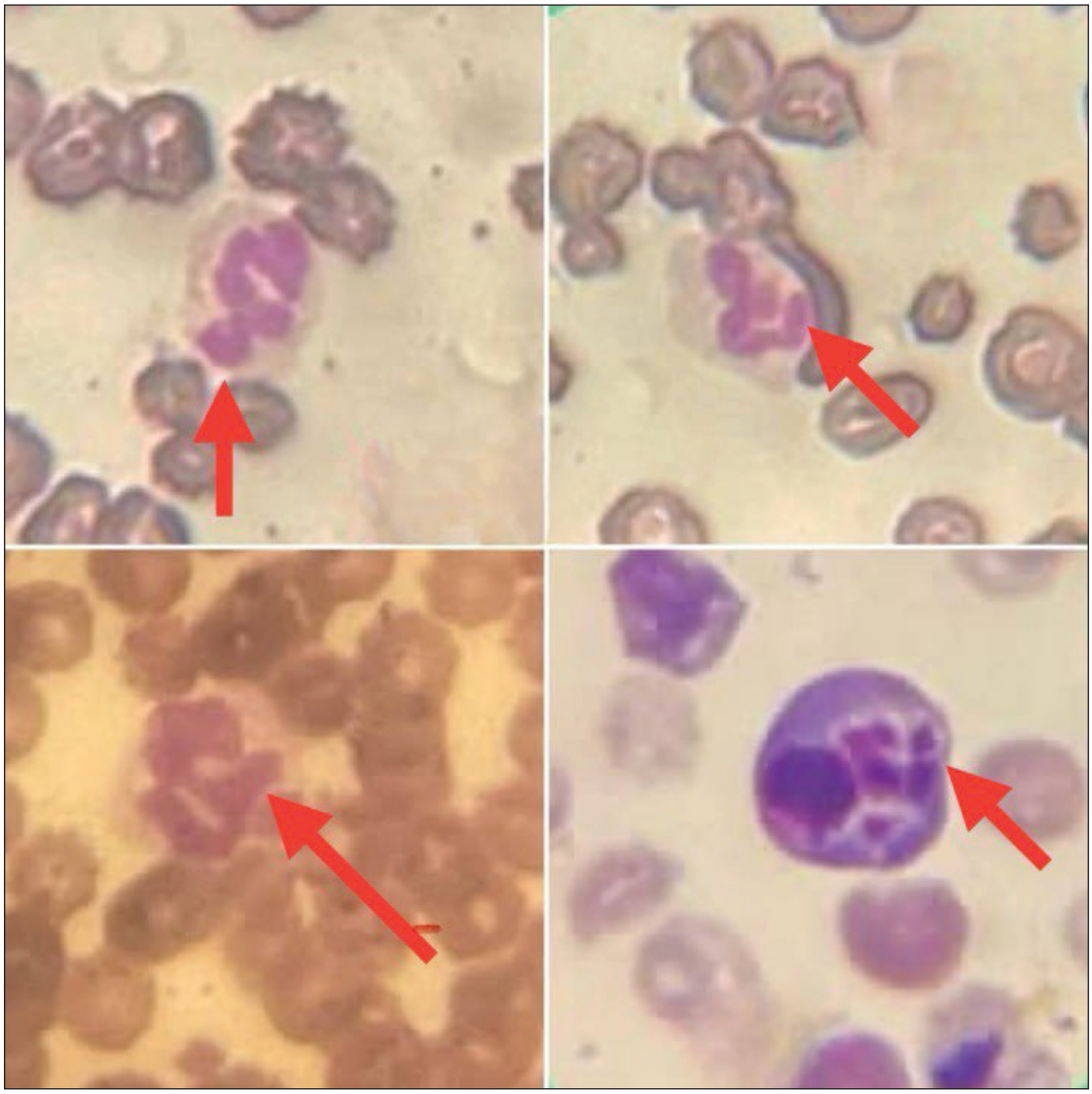

Fig. 3. Diff-Quik blood smears under an oil immersion lens showing morulae within leukocytes of dogs infected with *E. canis*.

Table 2. The haemato-biochemical profile in dogs with PCR-negative or PCR-positive *E. canis*.

	E. Canis		Mean difference	Reference interval	(*t*-test) f-value *p*-value
	PCR-negative	PCR-positive			
Hematological biomarkers					
RBC (10^{12}/l)	7.21 ± 0.98	4.52 ± 0.75	2.68	5.50–8.50	$F = 0.81$ $p \leq 0.00^{1**}$*
Hb (g/l)	144.06 ± 21.49	90.65 ± 11.56	53.41	120–180	$F = 4.05$ $p \leq 0.001^{***}$
PCV (%)	44.32 ± 7.44	27.17 ± 3.13	17.15	37–55	F=16.31 $p \leq 0.001$***
MCV (fl)	61.03 ± 4.05	61.33 ± 11.74	−0.30	60–77	F=7.17 $p = 0.93^{NS}$

(*Continued*)

Table 2. *Continued...*

	E. Canis		Mean difference	Reference interval	(*t*-test) f-value *p*-value
	PCR-negative	PCR-positive			
MCH (pg)	19.87 ± 1.09	20.25 ± 2.19	−0.39	19.49–24.49	F=8.55 p = 0.55^{NS}
MCHC (%)	32.65 ± 2.47	33.77 ± 5.32	−1.11	31.99–36.01	F= 3.39 p = 0.47^{NS}
WBC (10^9/l)	11.33 ± 1.34	14.13 ± 2.59	−2.80	6–17	F=5.37 $p \leq 0.001^{***}$
Neutrophil (10^9/l)	8.01 ± 0.88	9.23 ± 1.62	−1.23	3–11.8	F= 7.01 p = 0.014**
Lymphocyte (10^9/l)	2.30 ± 0.50	3.07 ± 1.09	−0.77	1–4.8	F=10.89 p = 0.018*
			(cont).		
Monocyte (10^9/l)	0.70 ± 0.12	1.45 ± 0.76	−0.75	0.15–1.35	F= 26.86 $p \leq 0.001^{***}$
Eosinophil (10^9/l)	0.32 ± 0.07	0.38 ± 0.06	−0.054	0.10–1.25	F=0.03 p =0.049*
Platelets (10^9/l)	227.36 ± 40.28	76.73 ± 22.60	150.63	150–500	F=1.61 $p \leq 0.00^{1**}$*
Biochemical indicators					
ALT (U/l)	73.38 ± 18.68	160.63 ± 12.85	−87.25	18–132	F=0.17 $p \leq 0.001^{***}$
AST (U/l)	37.57 ± 12.19	93.39 ± 7.88	−55.82	15–57	F= 0.84 $p \leq 0.001^{***}$
ALKP (U/l)	94.11 ± 34.16	287.28 ± 69.96	−193.17	21–170	F= 13.46 $p \leq 0.001^{***}$
γ-GT (U/l)	5.50 ± 0.89	9.51 ± 1.32	−4.01	1–6	F= 3.43 $p \leq 0.001^{***}$
Total. P (g/dl)	6.23 ± 0.62	7.27 ± 0.69	−1.05	5.5–7.1	F=0.63 $p \leq 0.001^{***}$
Albumin (g/dl)	3.28 ± 0.35	2.64 ± 0.42	0.64	2.9–3.7	F= 0.83 $p \leq 0.001^{***}$
Globulin (g/dl)	2.95 ± 0.56	4.63 ± 0.74	−1.68	2.4–3.8	F = 2.83 $p \leq 0.001^{***}$
A:G ratio	1.15 ± 0.27	0.59 ± 0.15	0.56	0.97–1.21	F = 5.16 $p \leq 0.001^{***}$
			(cont).		
T. BIL (mmol/l)	2.18 ± 0.32	7.86 ± 0.90	−5.68	1.7–6.8	F = 8.43 $p \leq 0.001^{***}$
Urea (mg/dl)	7.20 ± 0.90	17.44 ± 4.12	−10.24	8–25	F = 15.81 $p \leq 0.001^{***}$
Creatinine (mg/dl)	0.69 ± 0.13	1.76 ± 0.39	−1.06	0.6–1.4	F = 12.74 $p \leq 0.001^{***}$

Values are reported as mean ± SD. ALT: alanine transaminase, AST: aspartate transaminase, ALKP: alkaline phosphatase, Total. P: total protein, A: G ratio: albumin: globulin ratio, T. BIL: total bilirubin. Superscript NS indicates insignificant change, while the superscript (*) indicates significant change; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{****}p \leq 0.001$.

highest incidence of CME was observed in the group of dogs less than six months (42.4%, $p < 0.05$, OR = 12.526, 95% CI: 1.486–105.583), followed by the group of dogs between 6 months–1 year (38.3%, $p < 0.05$, OR= 10.568, 95% CI: 1.316–84.829), and the lowest incidence was in the group of dogs between > 3 years (5.6%). A significant association between the season and occurrence of CME was found, and the higher incidence was in the spring season (52.9%, $p = 0.001$, OR = 40.500, 95% CI: 4.472–366.775) followed by the summer (36.1%, $p < 0.01$, OR = 20.308, 95% CI: 2.602–158.471), and the lowest incidence was in the winter (2.7%). Another significant association between the tick infestation and the occurrence of CME was noticed, and the higher incidence was in dogs suffering

Table 3. shows the number and prevalence of positive cases with *E. canis* in different breeds.

Breeds	Stray group	Foreign breeds group					Total number
		Black German Shepherd	German Shepherd	Pit bull	Golden retriever	Grand Danois	
Number of dogs	48	132					180
	48	24	78	12	12	6	
Number and prevalence of positive dogs with *E. canis*	12 (6.66%)	6 (3.33%)	24 (13.33%)	0	0	0	

Table 4. shows the results of univariate and multivariate logistic regression.

Results of univariate logistic regression								
Variable	**Categories**	**Number of dogs (%)**	**Number of positive cases by PCR (%)**	**OR**	***P*-Value**	**CI**	β	**S.E.**
Age	(G1): < 6 months	33 (18.3)	14 (42.4)	12.526	0.020*	1.486—105.583	2.528	1.088
	(G2): 6 months- 1	60 (33.3)	23 (38.3)	10.568	0.027*	1.316–84.829	2.358	1.063
	year	69 (38.3)	4 (5.8)	1.046	0.969	0.110–9.980	.045	1.151
	(G3)1 - 3 years	18 (10)	1 (5.6)	0.059	0.006**	–	−−2.833	1.029
	(G4) > 3 years							
Breed	Stray	48 (26.67)	12 (25)	0.882	0.750	0.409–1.905	−0.125	0.393
	Foreign	132 (73.33)	30 (22.73)	0.333	0.001***	–	−1.099	0.333
Sex	Male	126 (70)	30 (23.8)	1.094	0.818	0.511–2.342	0.090	0.388
	Female	54 (30)	12 (22.22)	0.286	0.001***	–	−1.253	0.327
Season	Spring	17 (9.4)	9 (52.9)	40.500	0.001***	4.472–366.775	3.701	1.124
	Summer	61 (33.9)	22 (36.1)	20.308	0.004**	2.602–158.471	3.011	1.048
	Autumn	65 (36.1)	10 (15.4)	6.545	0.079	0.803–53.352	1.879	1.070
	Winter	37 (20.6)	1 (2.7)	0.028	0.001***	–	−3.584	1.014
Tick infestation	Yes	134 (74.4)	38 (28.4)	0.241	0.011*	0.081–0.717	−1.425	0.557
	No	46 (25.6)	4 (8.7)	0.396	0.001***	–	−0.927	0.192
Hemorrhage from natural orifices	Yes	14 (7.78)	7 (50)	0.267	0.020*	0.088–0.812	−1.320	0.567
	No	166 (92.22)	35 (21.1)	1.000	1.000	–	0.000	0.535
Location	El Dakahlia	77 (42.8)	25 (32.5)	2.432	0.014*	1.201–4.926	0.889	0.360
	El Gharbia	103 (57.2)	17 (16.5)	0.198	0.000	–	−1.621	0.265
Number of ectoparasitic treatment per year	Without treatment	31 (17.2)	20 (64.5)	32.727	0.001***	8.268–129.547	3.488	0.702
	One	26 (14.4)	14 (53.8)	21.000	0.001***	5.204–84.738	3.045	0.712
	Two	66 (36.7)	5 (7.6)	1.475	0.606	0.337–6.465	0.389	0.754
	Three or four	57 (31.7)	3 (5.3)	0.056	0.001***	–	−2.890	0.593
Presence of Morulae	Yes	66 (36.67)	39 (59.1)	0.019	0.001***	0.005–0.065	−3.979	0.636
	No	114 (63.33)	3 (2.6)	1.444	0.142	–	0.368	0.250

(*Continued*)

Table 4. *Continued...*

Results of multivariate logistic regression						
Variable	**Categories**	**OR**	***p*-value**	**CI**	β	**S.E.**
Age	(G1): < 6 months	12.526	0.020*	1.486—105.583	2.528	1.088
	(G2): 6 months- 1	10.568	0.027*	1.316–84.829	2.358	1.063
	year	1.046	0.969	0.110–9.980	0.045	1.151
	(G3)1 - 3 years	-	-	–	0[b]	–
	(G4) > 3 years					
Season	Spring	40.500	0.001***	4.472–366.775	3.701	1.124
	Summer	20.308	0.004**	2.602–158.471	3.011	1.048
	Autumn	6.545	0.079	0.803–53.352	1.879	1.070
	Winter	-	-	–	0[b]	–
Tick infestation	Yes	0.241	0.011*	0.081–0.717	–1.425	0.557
	No	-	-	–	0[b]	–
Hemorrhage from natural orifices	Yes	0.267	0.020*	0.088–0.812	–1.320	0.567
	No	-	-	–	0[b]	–
Location	El Dakahlia	2.432	0.014*	1.201–4.926	0.889	0.360
	El Gharbia	-	-	–	0[b]	–
Number of ectoparasitic treatment per year	Without treatment	32.727	0.001***	8.268–129.547	3.488	0.702
	One	21.000	0.001***	5.204–84.738	3.045	0.712
	Two	1.475	0.606	0.337–6.465	0.389	0.754
	Three or four	-	-	–	0[b]	–
Presence of morulae	Yes	0.019	0.001***	0.005–0.065	–3.979	0.636
	No	-	-	–	0[b]	–

S.E: Standard Error *$p < 0.005$; CI: confidence interval; OR: odds ratio; β: regression coefficient; G: group; the superscript (*) indicates significant change; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

from tick infestations (28.4%, $p < 0.05$, OR = 0.241, 95% CI: 0.081–0.717).

Also, hemorrhage from natural orifices was found to be significantly associated between the presence of the disease as the highest incidence occurred in dogs with bleeding (50%, $p < 0.05$, OR = 0.267, 95% CI: 0.088–0.812), the location had a role in the disease occurrence, and the prevalence was higher in El Dakahlia (32.5%, $p < 0.05$, OR = 2.432, 95% CI: 1.201–4.926) than El Gharbia (16.5), and also the number of ectoparasitic treatments had a significant association with the occurrence of CME, and the highest ratio was seen in the group of dogs that did not receive any ectoparasitic treatment per year (64.5%, $p = 0.001$, OR = 32.727, 95% CI: 8.268–129.547) followed by the group that received ectoparasitic treatment once per year (53.8%, $p = 0.001$, OR = 21.000, 95% CI: 5.204–84.738) while the lowest ratio was seen in the group of dogs that received ectoparasitic treatment three or four times per year (5.3%). It was found that there is a significant relation between the presence of morulae and the emergence of CME, and it was observed in the majority of infected dogs (59.1%, $p = 0.001$, OR = 0.019, 95% CI: 0.005–0.065).

Correlation of risk factors with haemato-biochemical findings

As visualized in Figure 4 and Table S1, the value of the Eta coefficient (η = 0.4–0.69) suggested a moderate correlation between the levels of RBCs, Hb, PCV, WBC, neutrophil, ALT, AST, creatinine, and all investigated risk factors. Conversely, RBCs indices (MCV, MCH, and MCHC) declare a weak or no association with these factors. Furthermore, η values for the presence of Morula in blood film were like that of season factor, tick infestation, and number of ectoparasitic treatments per year. As such, these risk factors declared a moderate correlation with the count of monocytes, eosinophils, and platelets as well as the levels of ALKP, γ-GT, total. P, globulin, A:G ratio T. BIL and urea. Whereas a weak or no correlation was detected between lymphocyte count, albumin levels, and these risk factors.

Likewise, age and hemorrhage from natural orifice showed a moderate association with the values of monocyte, ALKP, γ-GT, total. P, T. BIL, and urea. However, a weak correlation was detected with the count of lymphocytes, eosinophils, and platelets, together with the levels of albumin, globulin, and A:G ratio. Finally, the location of the sample collection showed either a weak or ineffective correlation with most variables.

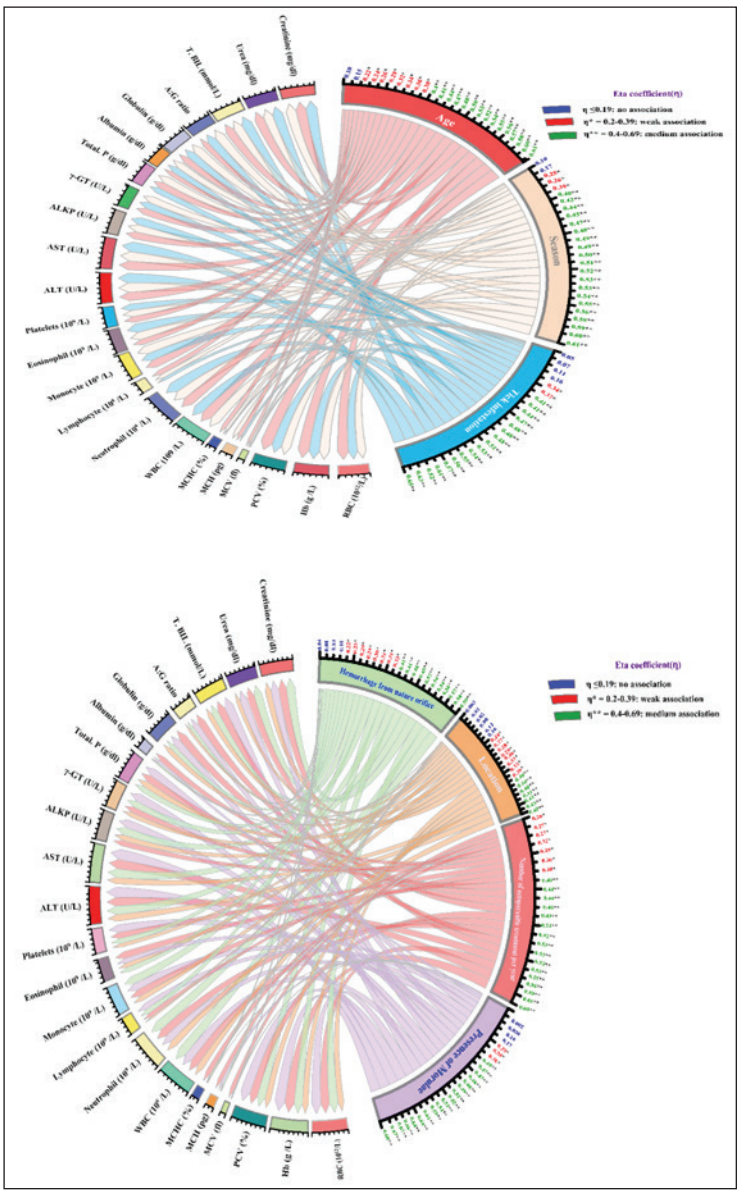

Fig. 4. Chord diagrams visually outline the intensity of the correlation between risk factors and the levels of haemato-biochemical variables in overall investigated dogs ($N = 180$). Links flow with colored nodes show the association between risk factors and blood variables. Risk factors are at the center of each node. Each link represents a haemato-biochemical parameter and parameters with no weak or strong association are visualized based on the width of the link. The numbers outside nodes reflect the calculated Eta coefficient (η) value for each parameter.

PCR

Out of 180 samples examined by PCR assay, 42 (23.33%) were positive for the genus of Ehrlichia (Fig. 5), and the same samples (42) were positive for the species-specific *E. canis* (Fig. 6). The prevalence of CME was (32.5%) in El Dakahlia and (16.5%) in El Gharbia.

Sequencing and phylogenetic analysis

By searching the National Center for Biotechnology Information (NCBI) database using a tool called BLAST to analyze the DNA sequence of the *E. canis* 16SrRNA gene and identify genes with similar sequences. The partial 16SrRNA gene (400 bp) of *E. canis* that was obtained in our study (GenBank

accession no. OR631919) had a nucleotide identity of 100% with other sequences that had been published before from Hong Kong (OP236554), Mexico (MG029069), USA (MH620197), India (OR359638), and Colombia (MT472811), while Egypt (MG564262), Egypt (MG564263), and Brazil (KC109445) shared 99.75% identity of nucleotide sequence, in addition to China that showed 99.02% nucleotide identity, as shown in the phylogenetic tree (Fig. 7).

Discussion

Canine vector-borne diseases (CVBDs) are of global importance (Nguyen *et al*., 2020). Among CVBPs, tick-borne Ehrlichia species are of great importance for dogs and pose a threat to public health as they can infect humans (Gokmen *et al*., 2019).

There are limited data available regarding the occurrence of CME in Egyptian dogs. In our study, the prevalence of *E. canis* was 23.33% in the two governorates, but the prevalence was more significant in El Dakahlia (32.5%) than in El Gharbia (16.5%), suggesting that the climate role in the multiplication of ticks while in 2015, the prevalence was 5.15% but based on detection of *E. canis* antibodies (Salib and Farghali, 2015). In 2019 and 2020, the prevalence was reported at 15.8% and 9.7%, respectively, based on PCR identification in other governorates in Egypt (Giza, Cairo, and Qalyubia) due to the veterinary care in these governorates (Abdelfattah *et al*., 2019; Selim *et al*., 2020). The higher prevalence rate based upon PCR was closely related to our results in Alexandria, Northern Egypt, which was 20% (El-Dakhly *et al*., 2021), in St. Kitts, West Indies was 27% (Kelly *et al*., 2013), in Pakistan was 28% (Malik *et al*., 2018) and in Senegal was 18.8% (Dahmani *et al*., 2019). The lower infection rates of *E. canis* were recorded at 1.7%–8% in Central Italy, 2.9% in Northern Italy, and 9.7% in Southern Italy (Ebani *et al*., 2015), and a higher prevalence rate was recorded in Colombia at 40.6% because *R. sanguineus* and *E. canis* are mostly found in Colombia (Vargas-Hernández *et al*., 2012).

In our study, infected dogs with *E. canis* showed multisystemic signs such as fever, lethargy, anemia, tachycardia, paleness of gum and conjunctiva, shivering, blindness, paralysis, hemoglobinuria, Melena, and some dogs died, and the prevalence of dogs which showed symptoms and PCR-positive was 13.33% (24/180), and out of 180, 18 dogs were asymptomatic and PCR-positive (10%), attributing to the subclinical phase CME as the infected dogs apparent healthy although the pathogen present in its blood and different tissues (Rodríguez-Alarcón *et al*., 2020).

The results during the examination of Diff-Quik blood smears in this study revealed the presence of morulae inside leukocytes in 66 dogs out of the total examined 180 (36.67%), and only 39 (59.1%) were positive for morulae and PCR-positive for *E. canis*. The presence of morulae and PCR-negative for *E. canis* in 27 samples (40.9%) may suggest infection with other pathogens related to the Anaplasmatacea family such as Anaplasma phagocytophilum, Anaplasma platys, and Neorickettsia risticii (Harrus and Waner, 2011; Sainz *et al*., 2015) or misdiagnosed with nuclear remnants of megakaryocytes and products raised from Platelet activation (Lara *et al*., 2020) or due to the artifacts during the staining process so the definite diagnosis must be by using PCR (El-Dakhly *et al*., 2021). On the other hand, the PCR-positive samples with the absence of morulae suggest a low level of *E. canis* in the bloodstream, even during the acute stage (Lara *et al*., 2020; Hegab *et al*., 2022).

The prevalence of CME was the highest in dogs less than six months 42.4% and the lowest percentage in the elderly dogs like that was reported (Malik *et al*., 2018; El-Dakhly *et al*., 2021) as puppies infested with ticks in their early stage of life or maybe the potential for *E. canis* to be passed from a pregnant bitch to its puppy through the placenta (Astigarraga, 2023; Sukara *et al*., 2023). This result conflicts with another study, which reported a higher incidence among elderly dogs aged more than five years (Selim *et al*., 2021).

There was no significant association between the breed and sex and the occurrence of CME, and this was agreed with (Selim *et al*., 2020). In contrast, previous studies recorded that the prevalence was higher in mixed breeds than in foreign purebreds due to the environmental condition that makes these dogs more susceptible to infestation of ticks and the less use of ectoparasitic treatment (Barrantes-González *et al*., 2016). Some published studies have found greater prevalence in males than females, which can be explained by the behavioral characteristics that make a chance of tick infestations (Costa Jr *et al*., 2007), while some other studies have found that the disease was most prevalent in female dogs (Selim *et al*., 2021).

Concerning the season, it has been found that the infection rate of CME reached its peak in the spring season (52.9%), followed by the summer season (36.1%), and our finding was closely related to (Selim *et al*., 2021) because the ticks' activation and multiplication take place from spring to the beginning of autumn, and the highest infestations occur during this time (Sainz *et al*., 2015). On the contrary, another study reported that the high prevalence of the Anaplasmataceae family was in summer, followed by spring (Hegab *et al*., 2022).

In our study, tick infestation was associated with *E. canis* infection, and this result agreed with previous studies that considered it as a risk factor owing to its great role as a main vector for CME transmission (Martínez-Vega *et al*., 2016; Selim *et al*., 2020). Moreover, some studies did not consider tick infestation a risk factor (Pérez-Macchi *et al*., 2019; Mitpasa *et al*., 2022).

Among the different clinical findings, the presence of hemorrhages from natural orifices like hemoglobinuria

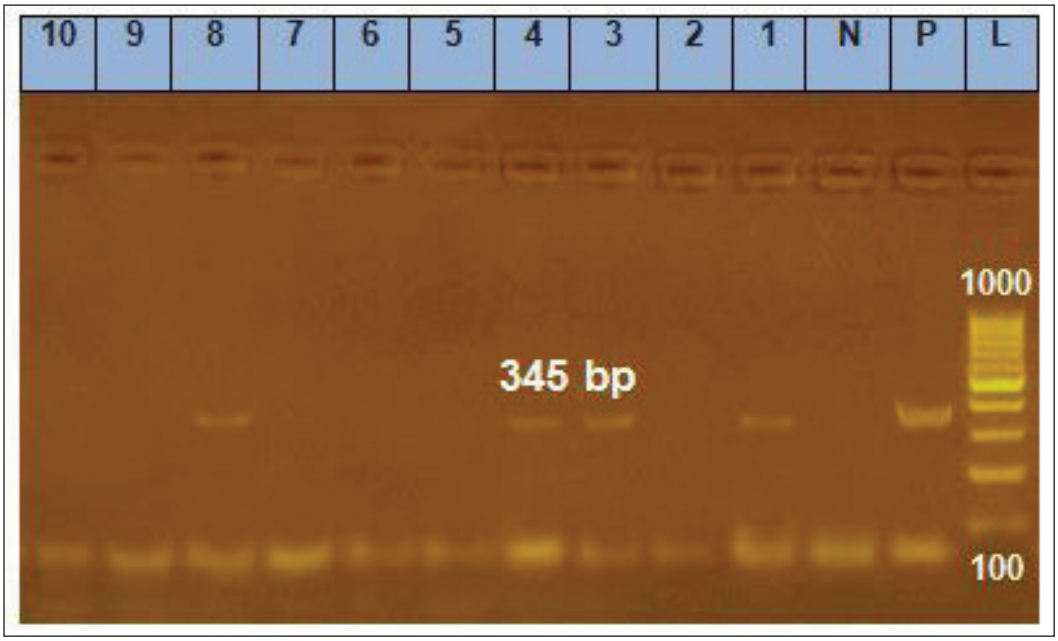

Fig. 5. Gel electrophoresis results for PCR of the genus of *Ehrlichia* gene, Lanes 1, 3, 4, 8 = positive samples; Lanes 2, 5, 6, 7, 9, 10 = negative samples; *N* = Negative control; P = Positive control; L= Ladder marker.

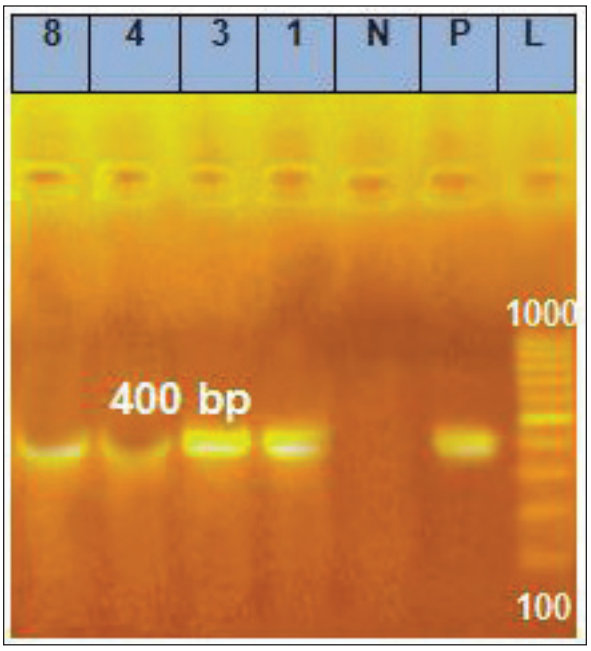

Fig. 6. Gel electrophoresis results for PCR of the same previous samples but by using the species-specific gene of *E. canis*, Lanes 1, 3, 4, 8=Positive samples; N=Negative control; P=Positive control; L=Ladder marker.

or melena was the most characteristic sign and observed in 50% of total infected dogs with *E. canis* based on PCR confirmation, so it was significantly associated with the disease since thrombocytopenia and immune complex deposition cause vascular wall damage (Salib and Farghali, 2015; Parashar *et al*., 2016). On the other hand, the results of the previous study indicated that these bleeding disorders were not associated with the occurrence of CME (Malik *et al*., 2018).

According to our findings, the highest frequency of CME 64.5% occurred in the dogs' group that did not receive any ectoparasitic treatment, either spray, shampoo, spot-on, or collar, which decreased the infestation of the different stages of ticks. The absence of veterinary care, sanitary measures, and the awareness of owners make them more susceptible to CME and other CVBDs as mentioned before (Selim *et al*., 2020; Selim *et al*., 2021) while the lowest frequency was 5.3% occurred in the dogs' group that received three or more ectoparasitic treatment per year suggested that the perfect veterinary care will eradicate these vectors (El-Dakhly *et al*., 2021).

Reported normocytic normochromic anemia and profound thrombocytopenia, which are considered the hallmark of ehrlichiosis in dogs confirmed to be naturally infected with *E. canis*, were in accordance with earlier findings (Harrus and Waner, 2011; Salem *et al*., 2014). In contrast to our results, (Bai *et al*., 2017) reported that hypochromic normocytic anemia was predominant among dogs positive for *E. canis*. In our study, hemorrhage from the natural orifice was moderately associated with RBCs and platelets count ($\eta > 0.39$). Henceforth, the authors agree with the recent findings (Espino-Solís *et al*., 2023)**,** who attributed the non-regenerative anemia and thrombocytopenia either

Fig. 7. A phylogenetic tree was created using nucleotide sequences of the *E. canis* 16SrRNA gene. The red rectangle indicates the sequence obtained in our study. The evolutionary relationships were inferred using a maximum likelihood method based on the MEGA 11 software. The analysis was then repeated 1,000 times to generate bootstrap values.

to the bone marrow depletion by *E. canis* associated hemorrhage or to the immune-mediated destruction of RBCs and platelets. Apart from this, (Harrus, 2015) stated that infection with *E. canis* usually enhances the systemic release of inflammatory mediators, which have an inhibitory action on bone marrow erythropoiesis and upregulate the migration and consumption of circulatory platelets at sites of tissue injury during inflammation.

The findings of our study declared that the leukogram pattern was within the normal reference intervals but showed a significant elevation of absolute WBC values and their fractions (neutrophil, lymphocyte, monocytes, eosinophil) in dogs with positive PCR results for *E. canis* when compared to those with negative PCR result. Similar findings have been reported recently by Gianopoulos *et al*., (2016), Malik *et al*. (2018), and Thongsahuan *et al*. (2020), who declared that these leukocyte changes are indicative of the acute stage of CME and infected dogs try to evade infection by enhancing the synthesis and release of leukocyte fractions at the central pool of blood in response to multiple inflammatory pathways. In contrast, many retrospective studies (Moreira *et al*., 2003; Assarasakorn *et al*., 2008; Chochlios *et al*., 2019) of naturally infected dogs demonstrated that significant decrease of leukocyte and neutrophil counts with a mild shift to the left of neutrophil. The finding of leukopenia in natural cases might reveal the development of mild transient inflammatory response concurrent with impaired neutrophil production, accelerated neutrophil utilization, or immune-mediated destruction of neutrophils (Waner *et al*., 2000; Harrus *et al*., 2001).

Considering hepatorenal indicators, hypoalbuminemia and enhanced activity of hepato-biliary enzymes (ALT, AST, ALP, and γ-GT) within the PCR-positive group are probably related to immune-mediated RBCs destruction, which causes ischemic necrosis

of hepatic tissue leading to enzyme leakage and decreased intravascular albumin mass. In earlier studies, this hypothesis was supported by finding different degrees of hepatocellular damage in dogs naturally infected with *E. canis* (Nicholson *et al*., 2010; Bai *et al*., 2017). In fact, albumin is a negative acute-phase protein and can be slightly decreased in response to acute tissue injury. However, in our study, the decrease in total. P is not observed due to the compensatory synthesis and release of globulins to act as positive acute phase reactants and trigger the inflammatory process. Furthermore, noted hypoalbuminemia in conjunction with high creatinine and urea levels suggests a transient renal impairment. A few recent studies (Ziliani *et al*., 2019; Chawla *et al*., 2020) supported our findings and reported the incidence of kidney failure in dogs positive for *E. canis*.

The phylogenetic analysis revealed that there is a clear relationship between the *E. canis* 16SrRNA gene of Egyptian isolate (OR631919) and other isolates submitted in GenBank, such as Hong Kong (OP236554), Mexico (MG029069), USA (MH620197), India (OR359638), and Colombia (MT472811) that shared 100% of nucleotide identity, in addition to Egypt (MG564262), Egypt (MG564263), and Brazil (KC109445) that showed 99.75% identity of nucleotide sequence and China with nucleotide identity of 99.02%. Our study confirmed the previous research that utilized the same *E. canis* 16SrRNA gene as a precise identification method (Wen *et al*., 1997; Gal *et al*., 2008).

The current study proves the existence of *E. canis* in El-Dakahlia and El-Gharbia governorates, and this is the first large-scale study concerning the epidemiological, clinicopathological examination, molecular characterization, sequencing, and phylogenetic analysis of reported from the center of the Delta of the Nile in Egypt.

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Conflict of interest

The authors of this paper have declared that no competing interests exist.

Authors' contribution

Mohamed Eisa and Samar atwa conceived and planned the study; Ahmed Selim and Dina Mobark collaborated in the writing and revision of the manuscript; Dina mobark and Ahmed sebaay conducted laboratory testing; Ahmed Selim and Elzahraa elbaz collaborated in sequencing of genes and phylogenetic analysis. Mohamed Eisa and Samar atwa revised the manuscript. All authors' read and endorsed the final manuscript.

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Data availability

All data are provided in the manuscript.

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Supplementary Materials

Table S1 outlines the obtained Eta values that inform the association degree between each risk factor and haemato-biochemical indicators. Calculated Eta-squared (η^2) that describes the amount of variance in each parameter that can be explained by one or more risk factors.

Table S1. Eta values for the correlation between risk factors and levels of haemato-biochemical variables in overall investigated dogs ($N = 180$).

	Age	Season	Tick infestation	Hemorrhage from nature orifice	Location	Number of ectoparasitic treatment per year	Presence of morulae
Hematological biomarkers				Nominal by interval [η (η^2)]			
RBC (10^{12}/l)	0.56 (0.31)	0.45 (0.20)	0.48 (0.23)	0.57 (0.32)	0.41 (0.17)	0.59 (0.35)	0.61 (0.37)
Hb (g/l)	0.58 (0.34)	0.52 (0.27)	0.55 (0.30)	0.54 (0.29)	0.41(0.17)	0.65 (0.42)	0.67 (0.45)
PCV (%)	0.61 (0.37)	0.49 (0.24)	0.51 (0.26)	0.53 (0.28)	0.41 (0.17)	0.53 (0.28)	0.64 (0.41)
MCV (fl)	0.15 (0.02)	0.097 (0.01)	0.072 (0.01)	0.039 (0.00)	0.003 (0.00)	0.26 (0.07)	0.036 (0.00)
MCH (pg)	0.22 (0.05)	0.26 (0.07)	0.11 (0.01)	0.098 (0.01)	0.011 (0.00)	0.27 (0.07)	0.16 (0.03)
MCHC (%)	0.24 (0.06)	0.17 (0.03)	0.051 (0.00)	0.075 (0.01)	0.081 (0.01)	0.32 (0.10)	0.002 (0.00)
WBC (10^9/l)	0.56 (0.31)	0.53 (0.28)	0.56 (0.31)	0.59 (0.35)	0.41 (0.17)	0.53 (0.28)	0.54 (0.29)
Neutrophil (10^9/l)	0.54 (0.29)	0.58 (0.34)	0.61 (0.37)	0.58 (0.34)	0.45 (0.20)	0.53 (0.28)	0.47 (0.22)
Lymphocyte (10^9/l)	0.29 (0.08)	0.25 (0.06)	0.16 (0.03)	0.29 (0.08)	0.12 (0.01)	0.27 (0.07)	0.38 (0.14)
Monocyte (10^9/l)	0.48 (0.23)	0.51(0.26)	0.48 (0.23)	0.48 (0.23)	0.33 (0.11)	0.44 (0.19)	0.45 (0.20)
Eosinophil (10^9/l)	0.34 (0.12)	0.48 (0.23)	0.53 (0.28)	0.29 (0.08)	0.29 (0.08)	0.44 (0.19)	0.29 (0.08)
Platelets (10^9/l)	0.36 (0.13)	0.40 (0.16)	0.41 (0.17)	0.31 (0.10)	0.24 (0.06)	0.40 (0.016)	0.48 (0.23)
Biochemical indicators							
ALT (U/l)	0.53 (0.28)	0.59(0.35)	0.61(0.37)	0.53(0.28)	0.43(0.18)	0.56(0.31)	0.64(0.41)
AST (U/l)	0.55(0.30)	0.60(0.36)	0.62(0.38)	0.54(0.29)	0.41(0.17)	0.61(0.37)	0.66(0.44)
ALKP (U/l)	0.44(0.19)	0.47(0.22)	0.47(0.22)	0.48(0.23)	0.36(0.13)	0.35(0.12)	0.52(0.27)
γ-GT (U/l)	0.40(0.16)	0.42(0.18)	0.41(0.17)	0.41(0.17)	0.28(0.08)	0.38(0.14)	0.52(0.27)
Total. P (g/dl)	0.41(0.17)	0.44(0.19)	0.34(0.12)	0.33(0.11)	0.40(0.16)	0.46(0.21)	0.49(0.24)
Albumin (g/dl)	0.26(0.07)	0.39(0.15)	0.37(0.14)	0.10(0.01)	0.023(0.00)	0.36(0.13)	0.17(0.03)
Globulin (g/dl)	0.38(0.14)	0.49(0.24)	0.44(0.19)	0.31(0.10)	0.3(0.09)	0.51(0.26)	0.48(0.23)
A:G ratio	0.32(0.10)	0.52(0.27)	0.48(0.23)	0.22(0.05)	0.16(0.03)	0.49(0.24)	0.38(0.14)
T. BIL (mmol/l)	0.47(0.22)	0.49(0.24)	0.54(0.29)	0.41(0.17)	0.33(0.11)	0.53(028)	0.59(0.35)
Urea (mg/dl)	0.49(0.24)	0.50(0.25)	0.54(0.29)	0.54(0.29)	0.27(007)	0.52(027)	0.47(0.22)
Creatinine (mg/dl)	0.54(0.29)	0.53(0.28)	0.57(0.32)	0.56(0.31)	0.4(0.16)	0.55(0.30)	0.51(0.26)

η indicates Eta coefficient; η^2 indicates Eta-squared. $\eta \leq 0.19$: no association; $\eta^* = 0.2–0.39$: weak association; $\eta^{**} = 0.4–0.69$: medium association; $\eta^{***} \geq 0.7–1$: strong association.