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RESEARCH ARTICLE

Prevalence and First Molecular Characterization of *Ehrlichia canis* in Egyptian dogs

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A B S T R A C T

Canine Ehrlichiosis is caused by *Ehrlichia canis*, an obligate intracellular bacterium. In Egypt, the epidemiology data about *Ehrlichia canis* is very limited and the molecular characterization of the organism has not been identified. Therefore, the objectives of this study were to study the prevalence of *E. canis* among 400 examined Egyptian dogs based on PCR assay and subsequently analyze the results by the logistic regression model. Furthermore, the molecular characterization of the Egyptian strain of *E. canis* was investigated. Generally, the prevalence of *E. canis* among dogs was 9.7%. The age, veterinary care, tick infestation, and antiparasitic treatment have a significant effect on the prevalence of *E. canis* in dogs as the prevalence rate was higher in older dogs (11.8%) and heavily infested dogs with ticks (10.3%) but lower in dogs received veterinary care (7.5%) and antiparasitic treatment (4.2%). In contrast, the sex, breed, and health status of dogs showed no significant role in the infection with *E. canis*. Sequence analysis of 16S rRNA for the Egyptian strain of *E. canis* revealed 100% identity with that of the American strain of *E. canis* and represented in one clade as obtained with a phylogenetic tree. The present study is the first study on molecular characterization of the Egyptian strain of *E. canis*, according to our knowledge.

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INTRODUCTION

Tick-borne Rickettsioses represent zoonotic and emerging diseases in Egypt (Selim *et al.,* 2018; Selim *et al.,* 2019a). Ehrlichiosis is an important one of them infecting dogs, caused by *Ehrlichia canis*, an obligatory gram-negative bacterium, that infects monocytes, granulocytes, and platelets. It is considered to be the main cause of Canine Ehrlichiosis (CE) in dogs (Kelly *et al.,* 2013; Lorsirigool *et al.,* 2020).

The infection with *E. canis* is more common during the summer season whereas ticks are more active. The organism transmitted mainly through various tick species such as the brown dog tick, *Rhipicephalus sanguineus* (Ansari-Mood *et al.,* 2015).

Prevention of ticks depends mainly on chemical pesticides and repellents for decades regardless of their effectiveness and of development of resistance, but using natural and safe materials as acaricides and repellents effectively controlled ticks (Khater *et al.,* 2013) and subsequently, prevent tick-borne diseases.

The clinical signs of CE can be divided into acute, subclinical, and chronic phases. The acute phase is characterized by fever, lymphadenopathy, epistaxis, and petechia (Neer and Harrus, 2006). During the subclinical phase, dogs appear healthy and could act as a carrier (Waner *et al.,* 1996). In chronic phase cases, an efficient immune response is not implemented by infected dogs and involved bone marrow resulting pancytopenia (Moreira *et al.,* 2005). Most of the dogs recover from the disease when treated with doxycycline or tetracycline with an adequate dose for the proper time.

Traditional diagnostic methods such as microscopic examination, serology, and isolation are reliable methods for the detection of CE (Ansari-Mood *et al*., 2015). The efficacy of blood smear examination to detect intracytoplasmic coccoid rickettsia inside monocyte is very limited because of the low level of parasitemia in most clinical cases. Moreover, the isolation of *E. canis* on cell culture is very sensitive and confirmatory method but it needs a long period (about one month) to give a result (Salib and Farghali, 2015). Also, the serodiagnosis for

Ehrlichiosis is very effective and reflect the number of antibodies in serum but it can't differentiate between early and late stage of infection (Ansari-Mood *et al*., 2015). Furthermore, the absence of antibodies against *E. canis* in the first two weeks of infections or cross-reaction with other Ehrlichial organisms increase the limitation of serological analysis (Moreira *et al.*, 2005).

Many studies have described PCR assay as the most reliable method for the detection of *E. canis* especially the nested PCR target 16SrRNA (Nakaghi *et al.*, 2010). On the other hand, the real-time PCR target disulfide bond formation protein (dsb) gene is more sensitive and specific to detect *E. canis* and differentiate it from other related bacteria (Labruna *et al.*, 2007). Therefore, it is supposed that the molecular technique is the most reliable method for the diagnosis of CE infection (Selim *et al.*, 2019a). The advantages of molecular detection attributed to the identification of the organism in the early stage before the development of antibodies and differentiation between *E. canis* and closely related *Ehrlichia* species using species-specific primers or sequencing (Selim and Gaede, 2015).

CE infections have been reported in dogs in Egypt by Salem *et al.* (2014); Salib and Farghali (2015) but the molecular characterization of the organism have not been determined. Therefore, the overall goals of this work were to investigate the prevalence of *E. canis* infections among domestic dogs and assess the associated risk factors besides the molecular characterization of *E. canis* strain among the Egyptian dogs.

MATERIALS AND METHODS

Ethics statement: Samples collection was performed under the verbal owner's consent. All procedures of the present research were performed according to ethical standards of the Faculty of Veterinary Medicine, Benha University whereas the sample was collected after physical constrains and followed the ethical guidelines

Samples collection and preparation: Blood samples were collected randomly from 400 dogs which were admitted to pet clinics in Cairo, Giza, and Qalyubia during 2018-2019. These Governorates are located at 30°2 N to 31°14 E , 30°01 N to 31°13 E and 30°25 N to 31°13 E. These areas have been selected based on the high density of pet animal population and geographic position (at the north of Egypt) as in Fig 1.

The weather of the selected area characterized by high temperatures (ranged between 25 to 40ºC) most of the years and rainy in winter. This weather allows multiplication and propagation of the ticks which consider the main vector for transmission of the disease. The samples were taken from clinically affected animals showed clinical signs such as fever, epistaxis, and vomiting as well as healthy inContact dogs.

Blood samples were drawn from the cephalic or saphenous vein of the dog on EDTA and were stored at -20°C until molecular examination. The data regarding the characteristic of examined dogs were obtained from owners include sex, age, breed, presence of ticks, and information about last antiparasitic treatment.

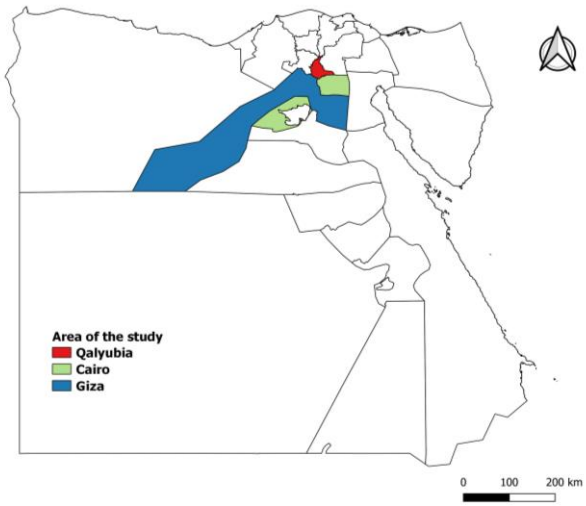

Fig. 1: Map of Egypt showing governorates of study.

DNA extraction: A total 400 blood samples were examined using conventional PCR assay. The DNA was extracted for whole blood of examined dogs using QIAamp® DNA Mini kit (QIAGEN GmbH, Hilden, Germany) followed the manufacturer's instructions. The extracted DNA was stored at -20ºC until being used in PCR assay.

PCR amplification: Conventional PCR was carried out for all examined samples using species-specific primers; forward CANIS 5′-CAA-TTA-TTT-ATA-GCC-TCT-GGC-TAT-AGG-A-3′ and reverse GA1UR 5′-GAG-TTT-GCC-GGG-ACT-TCT-TCT-3`to amplifies a 409 bp fragment of the 16S rRNA *E. canis* gene which previously evaluated by (Inokuma *et al.*, 2001).

The PCR reaction was carried out in a 25 µl reaction volume. The PCR master mix of single reaction included 1 µl of each primer (10 pmol/µl), 12.5 µl of Dream Taq Green PCR MasterMix (Thermo Fisher Scientific, US), and 5.5 µl of RNase-free water. Finally, 5.0 µl of DNA template was added. The thermal conditions were as follows; 1 cycle of 5 min at 95ºC, 40 cycles at 95ºC for 30 Sec, at 62ºC for 30 Sec, at 72ºC for 1 min, and final cycle of 5 min at 72ºC. The amplified PCR products were analyzed by 1.5% agarose gel.

Sequence and phylogenetic analysis: The PCR products of one positive sample were purified using the QIAPCR purification kit (QIAGEN GmbH, Hilden, Germany), following by direct sequencing using the ABI PRISM® BigDyeTM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

The obtained sequences were edited using BioEdit program (https://bioedit.software.informer.com/7.2/), followed by alignment with other published strains in GenBank using BLAST and constructed the phylogenetic tree using Mega7 software (https://www.megasoftware.net/) based on the neighbor-joining tree method with 1000 bootstrap replicates.

Statistical analysis: Data of the study were analyzed using the chi-square test using SPSS V24 (IBM, https://www.ibm.com/eg-en/products/spss-statistics) to determine the relationship between each variable and

prevalence of *E. canis* infection whereas the results considered significant if P-value ≤0.05. Logistic regression analysis was used to determine the effect of each variable on the prevalence of the disease.

RESULTS

Prevalence rate of *E. canis* in different localities: The results of molecular examination revealed that 39 out of 400 (9.7%) blood samples were positive for *E. canis*. The prevalence of *E. canis* infection showed non-significant variation (P=0.6) between different governorates. The highest rate was observed in Giza (11.8%), followed by Cairo (9.5%), and Qalyubia (8.3%) governorates, Table 1.

Risk factors associated with the prevalence of *E. canis*: Seven potential risk factors were evaluated using the logistic regression model, Table 2. The prevalence of Ehrlichiosis showed a non-significant difference between males (8.9%, 95% CI: 5.4- 14.1) and female dogs (10.5%, 95% CI: 6.8 – 15.7). According to the obtained results, the number of infected dogs varied significantly with age (P=0.01). The prevalence rate was increased in older dogs; 3.8%, (95% CI: 1.2 – 10) in dogs less than one-year-old and 11.8% (95% CI: 8.5 -16.2) in dogs more than one-year-old age. A further finding demonstrated a non-significant difference noted between the different breed of examined dogs as the prevalence rate was higher in German Shepherd (12.7%) in comparison with those of Rott weiler (5.5%) and Pit bull (6.6%).

Besides, the veterinary care, tick infestation and anti-parasitic treatment have a significant effect of prevalence of *E. canis* in domestic dogs whereas the veterinary care reduces significantly the percent of infected animals to (7.5%); the prevalence rate in periodical prophylactic treated dogs with anti-parasitic agents was significantly lower (4.2%, 95% CI: 2.2 – 9.7) than that of dogs had heavy infestation with ticks (10.13%, 95%CI: 7.3 – 13.7). Moreover, the health status of dogs had no significant role in the appearance of *E. canis* infection among dogs

Multivariate logistic regression analysis: Four risk factors have been assessed for the multivariant test. Significant odds ratios were observed regarding age, veterinary care, tick infestation, and anti-parasitic treatment, Table 3.

The odds ratio of age revealed that the prevalence rate in old dogs was 3.1 times higher than that of younger dogs. Moreover, the prevalence rate was decreased with veterinary care (OR=0.34, 95%CI: 0.18 -0.64) but increased with dogs have heavy tick infestation (OR=2.5, 95%CI: 0.36 – 17.6) and dogs had not been administered anti-parasitic treatment (OR=2.03, 95% CI: 1.02 – 4.06), Table 3.

Molecular characterization and phylogenetic analysis: The PCR products of one positive PCR sample were purified and sequenced. The obtained sequence was submitted to GenBank under accession number (LC490598). The BLAST analysis of the obtained sequences demonstrated 100% identity with *E. canis* strain from USA which published under AF546158 in GenBank but showed low homogeneity with other *E. canis* strain like U26740 from Israel. Also, *E. canis* strain

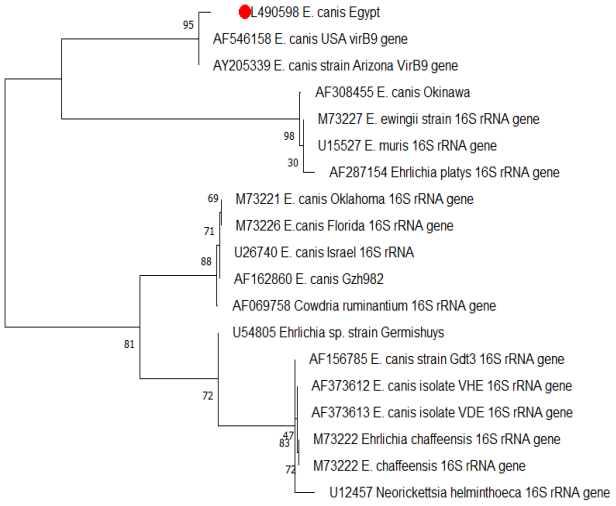

Fig. 2: Neighbor-joining tree of 16SrRNA gene showing phylogenetic relationship of *Ehrlichia canis* from the present study and other *Ehrlichia canis* strains available from GenBank.

Table 1: Prevalence of *Ehrlichia canis* among dogs in some Egyptian governorates

Locality	No. of examined	No. of positive	% of prevalence	95% CI	P value
Qalyubia	60	5	8.3	3.11 – 19.11	0.6*
Cairo	230	21	9.5	5.8 – 13.8	
Giza	110	13	11.8	6.6 – 19.7	
Total	400	39	9.7	7.2- 13.1	

*The results are non-significant at P>0.05.

Table 2: Logistic regression analysis for the association between each variable and prevalence of *Ehrlichia canis* infection

Variable	Total No.	positive	% of prevalence	95% CI	P value
Sex					
Male	191	17	8.9	5.4- 14.1	0.5
Female	209	22	10.5	6.8 – 15.7	
Age					
<1	105	4	3.8	1.2 – 10	0.01*
>1	295	35	11.8	8.5 -16.2	
Breed					
German Shepherd	220	28	12.7	8.7 – 18.09	0.085
Rott weiler	90	5	5.5	2.1 -13.1	
Pit bull	90	6	6.6	2.7 – 14.5	
Veterinary care					
Yes	345	26	7.5	5.1 – 10.9	0.0001*
no	55	12	21.8	12.2 – 35.4	
Tick infestation					
Yes	375	38	10.13	7.3 – 13.7	0.002*
No	25	1	4	0.2 – 22.3	
Anti-tick treatment					
Yes	235	10	4.2	2.2 – 9.7	0.02*
no	165	29	17.5	12.3 – 24.4	
Healthy status					
Healthy	335	33	9.8	6.9 – 13.6	0.87
Sick	65	6	9.2	3.8 – 19.6	

95% CI, 95% confidence interval; *The results are significant at p < 0.05

Table 3: Risk factors associated with seroprevalence of *Ehrlichia canis* infection in dogs

Risk factor	Comparative parameter	Odds ratio (OR)	95% CI
Age	<1	Ref	
	>1	3.1	1.1-8.5
Veterinary care	No	Ref	
	Yes	0.34	0.18-0.64
Tick infestation	Yes	2.5	0.36 – 17.6
	No	Ref	
Anti-tick treatment	No	2.03	1.02 – 4.06
	Yes	Ref	

95% CI, 95% confidence interval; OR, odds ratio.

of the current study genetically differed from other *Ehrlichia* species like *E. muris, E. ewingi,* and *E. platys*. Furthermore, the phylogenetic analysis showed that the *E. canis* strain (L490598) is closely related and present in one clade with *E. canis* USA (AF546158) and another strain from Arizona, Fig. 2.

DISCUSSION

The Egyptian dogs are suffering from many infectious diseases (Salib and Farghali, 2015) including canine ehrlichiosis which is a fatal tick-borne disease-inducing multisystemic disorders and generally appear as an acute disease with varying clinical signs (Guedes *et al.,* 2015). This is the first report regarding the molecular characterization of *E. canis* in dogs from Egypt.

The overall prevalence rate of *E. canis* in Egypt in the present work was 9.7% based on conventional PCR. A similar prevalence rate was recorded in Paraguay (10.41%). Higher prevalence was in Colombia (40.6%) (Vargas-Hernández *et al.,* 2012), Peru (51.3%) (Huerto-Medina and Dámaso-Mata 2015), and Costa Rica (32%) (Barrantes-González *et al.,* 2016), whereas lower prevalence was recorded in Europe (1.7-6%) (Ebani *et al.,* 2015) and Egypt (5.15%) (Salib and Farghali, 2015).

The prevalence rate of rickettsial infections was varied between different localities under the study indicating that the geographical factor and climatic conditions effect the distribution of tick-borne diseases (Mircean *et al.,* 2012; Selim and Ali, 2020).

These variations in the prevalence rate of *E. canis* worldwide may be contributed to density and population of the vector, socioeconomic factors, the difference in weather condition and the tests used for detection of the infection (Rodriguez-Vivas *et al.,* 2005; Selim *et al.,* 2019b).

An analysis of risk factors revealed that the positivity of *E. canis* increased with age and it was more frequent in older ones. These findings come in to agree with a previous study (Barrantes-González *et al*., 2016), which may be due to frequent exposure to *E. canis* infection throughout life (Stich *et al.,* 2014). In addition, the lack of veterinary care increased significantly the risk and rate of *E. canis* infection, because the absence of care and sanitary measures would increase the probability of infection with other bacterial, viral, and parasitic pathogens (Torres *et al.,* 2005).

Interestingly, the higher tick infestation in the examined dogs or the application of anti-parasitic treatment had a significant effect on the prevalence of *E. canis* in dogs. These findings may be contributed to the tick play a vital role in the transmission and spreading of the infection (Sainz *et al.,* 2015)

Furthermore, the sex, breed, and health status of dogs had no significant effect on the prevalence of *E. canis* infection as reported in similar findings by Derakhshandeh *et al.* (2017) and Malik *et al.* (2018).

The sequence analysis of *E. canis* strain from Egypt demonstrated high similarity with *E. canis* USA. This result confirms that the *E. canis* strains responsible for the mild and acute CE in Egypt and have identical 16S rRNA gene sequences indicating the worldwide distribution of *E. canis* (Siarkou *et al.,* 2007). To date, this is the first time for molecular characterization of *E. canis* strain in dogs from Egypt

Conclusions: *E. canis* was genetically characterized for the first time from dogs in Egypt, and further molecular studies and sequencing are needed to understand the epidemiology of the disease and to determine the presence of different species of Ehrlichia or other closely related genera in Egypt. Keen veterinary supervision especially for older dogs is crucial for reducing the infection. Furthermore, tick control with ecofriendly acaricides would prevent tick bites and their associated tick-borne diseases (Khater *et al.,* 2016).

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