ejpmr, 2020,7(10), 49-55

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

MOLECULAR DETECTION OF *EHRLICHIA CANIS* IN DOGS IN EGYPT

Esraa MA Khamis[1], Shwikar MA Ahmed[2], Dalia Talat[1] and Madiha Salah Ibrahim[1*]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt.
[2]Department of Medical Microbiology and Immunology, Faculty of Medicine, Alexandria University, Alexandria, Egypt.

***Corresponding Author: Madiha Salah Ibrahim**

Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt.

Article Received on 30/07/2020 **Article Revised on 20/08/2020** **Article Accepted on 10/09/2020**

ABSTRACT

Canine Ehrlichiosis is a fatal tick-borne disease in dogs transmitted by brown tick called Ripicephallus Sanguinus. Canine monocytic Ehrlichiosis is an intracellular gram-negative bacterial infection with 3 clinical phases: acute, subclinical, and chronic. Clinically, the dog infected with Ehrlichia shows common symptoms as fever, sever emaciation, off food, redness of conjunctiva. Here, blood samples were collected from 46 dogs (37 suspected with Ehrlichia) and 9 cases normal control from 3 Governmental veterinary units and 3 private clinics (24 males and 22 females) in Egypt. Out of the 37 suspected cases, 15 were feverish, 7 were off food, 15 appeared emaciated, 15 showed thrombocytopenia, and one case had epistaxis and died within 72 hours. By Nested PCR for the 46 cases, 17 (37.0%) were positive, while, 22 (47.8%) cases showed morulae in the monocytes. Nested PCR is more sensitive and accurate test confirming *E. canis* infection, however, blood smear testing could be used as a cheap, rapid routine diagnosis for dogs infested with ticks. The current work is the second for the detection of *E. canis* 16S RNA gene by Nested PCR in Egypt, further surveillance studies are still required to monitor *E. canis* infection in dogs in Egypt.

KEYWORDS: *E. canis*, dogs, morulae, Egypt.

1. INTRODUCTION

Canine Monocytic ehrlichiosis (CME) is a fatal tick-borne disease worldwide. Canine ehrlichiosis has emerged as one of the most important infectious diseases affecting dogs **(Moreira et al., 2003)**, infecting mainly macrophages, monocytes and granulocytes **(Jadhav et al., 2011)**.

Canine Ehrlichiosis is transmitted by brown tick called Rhipicephalus Sanguineus **(Nazari et al 2013)**. Following an incubation period of 8-20 days, Canine Ehrlichiosis has three clinical phases; acute, subclinical, and chronic phases. Dogs in acute phase respond rapidly to treatment and show great improvement in symptoms but **2.** untreated dogs recover from acute phase after 2-4 weeks to enter subclinical phase, which lasts from months to years a showing healthy state but could infect another dog. Chronic phase gives poor prognosis, not responding to treatment, and characterized by high mortality rate due to severe bleeding **(Harrus and Waner, 2011)**. Definitive diagnosis is based on microscopic examination of blood smears searching for morulae and detection of DNA by Polymerase chain reaction (PCR). Serological diagnosis fails to differentiate between current infection and previous one or exposure without establishment of infection **(Allison and Little, 2013)**.

Polymerase chain reaction (PCR) is a sensitive method of the detection of acute monocytic ehrlichiosis in dogs, even before the onset of clinical signs. Moreover, it can detect *Ehrlichia canis* (*E. canis*) before the development of antibodies in the early stages of the disease and identify new species using species-specific primers **(NAKAGHI et al., 2008).**

Therefore, this study was undertaken to detect *E. canis* DNA from blood samples of dogs by nested PCR and blood smear rapid testing aiming for early and rapid diagnosis of canine monocytic ehrlichiosis.

MATERIALS AND METHODS Blood samples

Blood samples were collected twice from 46 dogs (37 suspected having ehrlichiosis and 9 control apparently normal dogs) from 3 private and 3 governmental veterinary clinics in Egypt. Selection was random based on the willingness of the owners to have blood sample from their dogs. A questionnaire was filled at the sampling site in order to gather data (age, sex, breed, body temperature, mucous membrane status, the presence of haematuria, off food and tick infestation) and to report risk factor(s) associated with the prevalence of *E. canis* DNA in dogs, if any. The body of each dog, with special attention to the ears, was examined for the presence of ticks. Peripheral blood smears were made

and stained with Giemsa stain for microscopic examination (**Vaden et al., 2009**).

Polymerase chain reaction (PCR)

Whole blood samples were collected in tubes with EDTA for DNA isolation, stored at 4^{o}C in ice box until transferred to laboratory **(Selim et al., 2020).** Genomic DNA was extracted with Quick-gDNA Blood Mini Kit (ZYMO RESEARCH), according to the manufacturer's recommendations. The Ehrlichia genus amplification was performed using ECC (5'-GAACGAACGCTGGCGGCAAGC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGCA - 3') primers, and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT - 3') and ECAN (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3') primers were used to amplify the *E. canis* 16S rRNA gene **(Gal et al., 2008; Inokuma et al., 2003;and Inokuma et al., 2001).** Nested PCR was carried out in a thermal cycling procedure was; 1 cycle of 1 minute at 94^{o}C, 30 cycles of 1 minute at 94^{o}C, 2 minute at 65^{o}C, 2 minutes at 72^{o}C, and final cycle of 5 minutes at 72^{o}C **(Nazari et al 2013)**. The amplification products were visualized on a 2% agarose gel after electrophoretic migration at 100 voltages.

3. RESULTS

Out of the 46 dogs, 37 was suspected and 9 control dogs, 24 (52.2%) were males and 22 (47.8%) females with mean age 1.75±1.31 years old. Twenty-two cases (47.8%) were Balady (mixed breed), 9 (19.6%) German Shepard, 6 (13%) Wolf, 4 (8.7%) Golden Retriever, 3 (6.5%) Husky,1 (2.2%) Mastive and 1 (2.2%) boxer. Out of the 46 dogs, 35 (76.1%) were infested with ticks (Table 1).

The 37 suspected cases showed at least one symptom at the time of examination. They showed different manifestations of the disease as fever in 15 cases (32.6%), emaciation in 15 cases (32.6%), off food in 7 cases (15.2%), thrombocytopenia in 15 cases (32.6%). The 9 (19.6%) control dogs showed no symptoms and where clinically normal (Table 1). By microscopic examination of the blood smears for all dogs, 22/46 (47.8%) were positive for *Ehrlichia canis* morulae; 11 males (50%), 11 females (50%), while, 24/46 were negative (52.2%); 13 males (54.2 %), and 11 females (45.8%) as shown in Table 1 and figure 1. The mean age of positive cases was 1.69±1.31 years as compared to mean age of negative cases of 1.79±1.33 years.

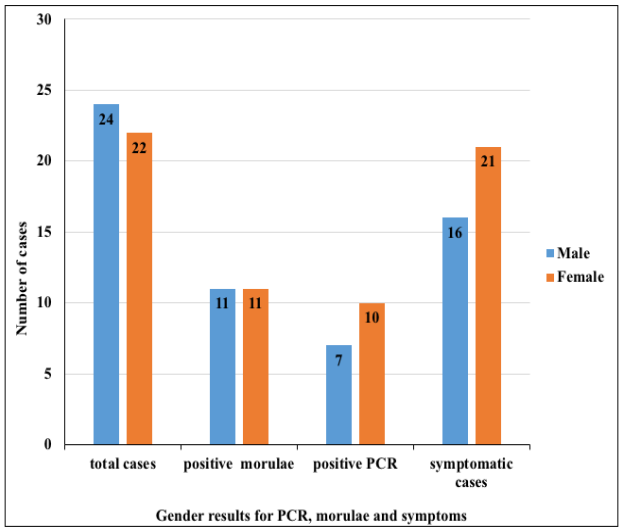

Figure 1: Total number of dogs, gender, dogs with morulae, PCR positive dogs and cases showing clinical manifestations.

For positive blood smear cases; the majority were Balady mixed breed; 12 cases (54.5%), 6 German Shepherd (27.3%), 2 Golden retrievers (9.1%), 1 Husky (4.55%) and 1 Wolf (4.55%). Out of those positive cases, 20 dogs (90.9%) were tick-infested and two dogs were not (9.1%). One of the asymptomatic dogs was a Balady one and was blood smear positive (Table 1).

Regarding the clinical manifestations of the positive blood smear cases, 21 (95.5%) dogs showed symptoms of fever, emaciation, off food and thrombocytopenia.

For negative blood smear cases, the majority were Balady mixed breed; 10 cases (41.7%), 3 German Shepherd (12.5%), 5 Wolf (20.8%), 2 Golden retrievers (8.3%), 2 Husky (8.3%), 1 Mastive (4.2%) and 1 Boxer (4.2%). Also, 15 dogs (62.5%) were tick-infested and 9 dogs were negative for ticks (37.5%). The negative blood smear cases showed clinical symptoms in 16 dogs (66.7%) and 8 asymptomatic cases were negative as well.

By comparing blood smear positive and negative cases, there was statistically significant difference between symptomatic cases and asymptomatic cases (P-value 0.021) as well as tick infestation (P-value 0.025) (Table 1).

By PCR amplification, 398 bp fragment from 16S rRNA gene of *E. canis*, was detected in 17 (37%) cases. There were statistically significant differences between the PCR positive and negative groups (P-value < 0.05) specially in the presence of fever and symptomatic and asymptomatic cases. One asymptomatic case was PCR positive and was tick infested as well (Table 1).

E. canis 16S rRNA gene was detected in 7 males (41.2%) and 10 females (58.8 %). The majority were Balady dogs (n=7; 41.2%), 4 German shepherd (23.5%), 3 Husky (17.6%), 2 Golden retriever (11.8%) and 1 wolf

(5.9%). Fifty percentage of the PCR positive cases were morula negative, while only 38% of the morula positive cases were PCR positive.

Tick infestation was detected in 76.1% of all dogs with higher percentage in the symptomatic cases (91.9%) as compared to the control asymptomatic cases (11.1%).

Table (1): Collective results for all samples for the blood smear and PCR in relation to number of cases, gender, age, breed, clinical manifestations and tick infestation.

Character/test	No. (%)	Morula +ve No. (%)	Morula –ve No. (%)	PCR +ve No. (%)	PCR –ve No. (%)	Symptomatic No. (%)	Asymptomatic No. (%)
Total No. (%)	**46 (100)**	**22 (47.8)**	**24 (52.2)**	**17 (37)**	**29 (63)**	**37 (80.4)**	**9 (19.6)**
Mean Age (Years)	1.75±1.31	1.69±1.31	1.79±1.33	2.011±1.377	1.59±1.264	1.84±1.388	1.33±0.849
Gender							
Male	24 (52.2)	11 (50)	13 (54.2)	7 (41.2)	17 (58.6)	16 (43.2)	8 (88.9)
Female	22 (47.8)	11 (50)	11 (45.8)	10 (58.8)	12 (41.4)	21 (56.8)	1 (11.1)
Breed							
German shepherd	9 (19.6)	6 (27.3)	3 (12.5)	4 (23.5)	5 (17.2)	9 (24.3)	0
Husky	3 (6.5)	1 (4.55)	2 (8.3)	3 (17.6)	0	3 (8.1)	0
Golden retriever	4 (8.7)	2 (9.1)	2 (8.3)	2 (11.8)	2 (6.9)	4 (10.8)	0
Balady	22 (47.8)	12 (54.5)	10 (41.7)	7 (41.2)	15 (51.7)	20 (54.1)	2 (22.2)
Wolf	6 (13)	1 (4.55)	5 (20.8)	1 (5.9)	5 (17.2)	1 (2.7)	5 (55.6)
BX	1 (2.2)	0	1 (4.2)	0	1 (3.5)	0	1 (11.1)
MS	1 (2.2)	0	1 (4.2)	0	1 (3.5)	0	1 (11.1)
Clinical Manifestations							
Symptomatic	37 (80.4)	21 (95.5)	16 (66.7)	16 (94.1)	21 (72.4)	+	-
Fever	15 (32.6)	9 (40.9)	6 (25)	9 (52.9)*	6 (20.7)*	+	-
Emaciation	15 (32.6)	8 (36.4)	7 (29.2)	8 (47.1)	7 (24.1)	+	-
Off Food	7 (15.2)	4 (18.2)	3 (12.5)	3 (17.6)	4 (13.8)	+	-
Thrombocytopenia	15 (32.6)	8 (36.4)	7 (29.2)	7 (41.2)	8 (27.6)	+	-
Asymptomatic	9 (19.6)	1 (4.5)*	8 (33.3)*	1 (5.9)*	8 (27.6)*	-	-
Ticks							
Positive	35 (76.1)	20 (90.9)	15 (62.5)*	14 (82.4)	21 (72.4)	34 (91.9)	1 (11.1)
Negative	11 (23.9)	2 (9.1)	9 (37.5)*	3 (17.6)	8 (27.6)	3 (8.1)	8 (88.9)

*There is a statistically significant difference between the 2 groups (P-value < 0.05).

Table (2): Comparison between blood smear and PCR results in relation to gender, breed, clinical manifestations and tick infestation.

Character/test	Blood Smear -ve /PCR -ve No (%)	Blood Smear +ve / PCR +veNo (%)	Blood Smear +ve /PCR -ve No (%)	Blood Smear -ve /PCR +ve No (%)
No.	8	8	13	8
Mean Age (Years)	1.8±1.32	2.0±1.48	1.5±1.26	2.2±1.46
Gender				
Male	3 (37.5)	3 (37.5)	7 (53.8)	3 (37.5)
Female	5 (62.5)	5 (62.5)	6 (46.2)	5 (62.5)
Breed				
German shepherd	2 (25)	3 (37.5)	3 (23.1)	1 (12.5)
Husky	0	1 (12.5)	0	2 (25)
Golden retriever	1 (12.5)	1 (12.5)	1 (7.7)	1 (12.5)
Balady	5 (62.5)	2 (25)	9 (69.2)	4 (50)
Wolf	0	1 (12.5)	0	0
Clinical Manifestations				
Fever	2 (25)	5 (62.5)	4 (30.8)	4 (50)
Emaciation	2 (25)	3 (37.5)	5 (38.5)	5 (62.5)
Off Food	2 (25)	2 (25)	2 (15.4)	1 (12.5)
Thrombocytopenia	3 (37.5)	3 (37.5)	5 (38.5)	4 (50)
Ticks				
Positive	7 (87.5)	7 (87.5)	13 (100)	7 (87.5)
Negative	1 (12.5)	1 (12.5)	0	1 (12.5)

DISCUSSION

E. canis has been detected and reported in dogs from many parts of the world **(Harrus et al., 2011; Rani et al., 2011; Sasaki et al., 2012; Ybañez et al., 2012; Nazari et al., 2013; Aktas et al., 2015; Inpankaew et al., 2016; Abdelfattah et al., 2019)**.

In the present study, *E. canis* was successfully detected using nested PCR technique and this represents the second molecular detection of this pathogen in Egypt where the first was reported by **Selim et al., (2020).**
The results of the present study showed that PCR is more sensitive than routine microscopic examination of blood smear in the detection of early infection as reported earlier by **Lakshmanan et al., (2007)**. These results agree with **Derakhshandeh et al., (2017)** who found it difficult to detect morulae through microscopic examination as they found it in only 4% of their analysed cases, while in our study more than 40% of the cases were morulae positive.

There are variations between different studies mainly in seasonality, breeds, age, diagnostic methods and geographical areas **(Milanjeet et al., 2014)**.

Here, the common prevalence was detected in dogs from 1.3 -2 years old, true positive for the presence of morulae and positive for PCR, which is consistent with several studies stating that the older the dog the greater the probability of exposure to infection **(Pinter et al., 2008; Vieira et al., 2013)**.

The high prevalence was detected in German Shepard (37.5%) as shown in Table 2 similar to that reported by **Abdelfattah et al., (2019)** who showed that 23.5% of the dogs were German shepherded. Balady breed showed 25% prevalence of infection positive for the presence of morulae and positive for PCR similar to results by **Derakhshandeh et al., (2017)**. Presence of ticks could be a proof for *E. canis* infection, especially with the mixed breeds (balady) where they are not exposed to medical treatment or attention as with pure breeds. This managemental factor may explain why pure breeds could be protected against *E. canis* as most infections were in mixed breed exposed to ticks **(Alexander et al.,2016),** in contrast to **Harus et al., (2004)** where the prevalence rates were low in both clinic cases and stray dogs. Further, the highest prevalence of *E. canis* was detected in stray dogs while the lowest were observed in Labradors (**Salem et al., 2013; Malik et al., 2018**).

Ehrlichia species multiply in the blood cells and form intracytoplasmic microcolonies called morulae during the acute phase of infection **(Mcquiston et al., 2003**). Some studies have found higher seropositivity in males, but this may be explained by a higher exposure to vectors than females, due to behavioral characteristics **(Costa et al., 2007)**.

In the study of **Derakhshandeh et al., (2017)**, canine ehrlichiosis was observed only in female dogs, which agrees with our study as the number of symptomatic and positively infected females was higher than males as reported by **Milanjeet et al., (2014),** while, in contrast to **Solano-Gallego et al., (2006)** and **Maazi et al., (2013)** findings. The reasons for such low detection rates in males require excessive investigation, however, it is important to consider that subclinical and chronic Ehrlichial infections are not as readily diagnosed as acute infections when canine blood is used for the detection of *E. canis*. Therefore, ideally, PCR using both blood and splenic aspirates should be considered to overcome this limitation. Thus, as found here 8 PCR positive samples were blood smear negative indicating that PCR could detect early infection before clinical symptoms as shown in Table 2.

With PCR negative samples (13 samples) despite their blood smears were positive for the presence of morulae as shown in Table 2, it may possibly indicate infection with some closely related pathogens such as *Ehrlichia ewingii*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum* and *Neorickettsia risticii*, which were shown to cause the same clinical and haematological manifestations in dogs as *E. canis* **(Mojgan et al., 2013)**.

There were 8 dogs negative in both PCR and blood smear indicating freedom from *E. canis* infection. Other 8 dogs were positive in both test, which indicates a good correlation for selection criterion of dogs in relation to their clinical manifestation and data recorded from owners. Further, this also implies that PCR is more accurate for detecting *E. canis* **(Breitschwerdt et al., 1998; Sainz et al., 2015).**

There was no significance between seasons in Egypt for the detection of *E. canis*, although **Mosallanejad et al., (2010)** reported a highest prevalence in summer. Ticks became more adaptive due to global climatic changes **(Leschnik et al., 2008)**, and the dynamics of tick prevalence depends on such climatic conditions followed by changes in seasonal patterns **(Friedhoff, 1988)**. In Egypt, the weather is warm throughout the year, which may have an impact on the pattern of tick prevalence in Egypt. Here, tick infestation was detected in 35/46 (71.4%) dogs indicating a high susceptibility to infection with not only *E. canis* but also any other tick-born infections. Further, *E. canis* was detected in 87.5% of the tick-infested dogs from cases positive morulae and positive PCR (Table 2) indicating a direct correlation between infection and tick detection **(Costa et al., 1973; Botros et at., 1995; Neer et al., 2002; Khazeni et al., 2014 and Stich et al., 2014).** Moreover, coinfection must be considered as may be the presence of morulae could be due to infection(s) other than *E. canis* **(Rojaset al., 2014; Wei et al., 2014).**

The prevalence of *E. canis* varied significantly ($P < 0.001$) between three sampling sites from Pakistan in 2018 as reported by **Malik et al., (2018)** that can be due

to different climatic conditions that affects the prevalence of ticks and hence *E. canis* prevalence. These variation in the prevalence of *E. canis* could be due to many factors including the distribution and population density of the vector **(Otranto et al. 2011)**, the sampling methodology and the characteristics of the targeted dog population **(Gomes et al. 2010; De Miranda et al. 2014)**. These results indicate that *E. canis* is prevalent in dogs in Egypt. Dogs infested with ticks with fever, emaciation, off food should be diagnosed and treated as soon as possible to avoid entering in subclinical or chronic phase, as it is a fatal disease. Further accurate and wider surveillance studies are required to understand the prevalence of *E. canis* and other infections in companion animals to be able to develop control and preventive strategies.

REFERENCES

1. **Vieira, R.F., Vieira, T.S., Nascimento D. do, A., Martins, T.F., Krawczak, F.S., Labruna, M.B., Chandrashekar, R., Marcondes, M., Biondo, A.W., Vidotto, O.** Serological survey of Ehrlichia species in dogs, horses and humans: zoonotic scenery in a rural settlement from southern Brazil. Rev. Inst. Med. Trop. SaoPaulo, 2013; 55: 335–340.
2. **RicaAlexander V. Barrantes-González, Ana E. Jiménez-Rocha, Juan José Romero-Zuˇniga,Gaby Dolz. 2016.** Serology, molecular detection and risk factors of Ehrlichia canis infection in dogs in Costa Rica. Elsevier. Ticks and Tick-borne Diseases, 2016; 7: 1245–1251.
3. **Rojas, A., Rojas, D., Montenegro, V., Gutiérrez, R., Yasur-Landau, D., Baneth, G.** Vector- borne pathogens in dogs from Costa Rica: first molecular description of Babesia vogeli and Hepatozoon canis infections with a high prevalence of monocytic ehrlichiosis and the manifestations of co-infection. Vet. Parasitol, 2014; 199: 121–128.
4. **Wei, L., Kelly, P., Ackerson, K., Zhang, J., El-Mahallawy, H.S., Kaltenboeck, B., Wang, C.** First report of Babesia gibsoni in Central America and survey forvector-borne infections in dogs from Nicaragua. Parasites Vectors 2014; 7: 126.
5. **Mosallanejad, AR; Jalali, BR and Alborzi, MH.** Sero-prevalence of Ehrlichia canis in dogs referred to veterinary hospital of Shahid Chamran University of Ahvaz, Iran, 2010; 65: 21-26.
6. **Leschnik, M; Kirtz, G; Tichy, A and Leidinger, E**. Seasonal occurrence of canine babesiosis is influenced by local climate conditions. Int. J. Med. Microbiol, 2008; 298: 243-248.
7. **Friedhoff, KT** Transmission of Babesia. Babesiosis of domestic animals and man. 1st Edn., Boca Raton, USA, CRC Press, 1988; 23-52.
8. **Mojgan Nazari, Sue Yee Lim, Mahira Watanabe, Reuben S. K. Sharma, Nadzariah A. B. Y. Cheng, Malaika Watanabe.** Molecular Detection of Ehrlichia canis in Dogs in Malaysia. PLoS Negl Trop Dis, 2013; 7(1): e1982.
9. **Harrus S, Kenny M, Miara L, Aizenberg I, Waner T, et al.** Comparison of simultaneous splenic sample PCR with blood sample PCR for diagnosis and treatment of experimental Ehrlichia canis infection. Antimicrobial Agents and Chemotherapy, 2004; 48: 4488.
10. **Jadhav, RK; Kumari, RR; Javed Jameel, A and Kumar, P** Emergence of arthropod transmitted infections in kennel dogs. Vet. World, 2011; 4: 522-528. doi:10.5455/vetworld.2011.522-528.
11. **Moreira, SM; Bastos, CV; Araújo, MS and Passos, LMF.** Retrospective study of canine ehrlichiosis in Belo Horizonte of canine ehrlichiosis in Belo Horizonte. Brasileoro Archive of Veterinary Medicine and zooteenia, 2003; 55: 141- 147**.**
12. **Nazari M, Lim SY, Watanabe M, Sharma RSK, Cheng NABY, Watanabe M**. Molecular Detection of *Ehrlichia canis* in Dogs in Malaysia. PLoS Negl Trop Dis, 2013; 7(1): 1982. https://doi.org/10.1371/journal.pntd.0001982.
13. **Lakshmanan, B; John, L; Gomathinayagam, S and Dhinakarraj, G. Molecular** detection of Ehrlichia canis from blood of naturally infected dogs in India. Vet. Archiv, 2007; 77: 165- 166.
14. **Schultz, R.M., Johnson, E.G., Wisner, E.R., Brown, N.A., Byrne, B.A., Sykes, J.E.**. Clinicopathologic and diagnostic imaging characteristics of systemic aspergillosis in 30 dogs. J. Vet. Intern. Med, 2008; 22: 851–859.
15. **Nyindo, M., Huxsoll, D.L., Ristic, M., Kakoma, I., Brown, J.L., Carson, C.A., Stephenson, E.H.** Cell-mediated and humoral immune responses of German Shepherd Dogs and Beagles to experimental infection with Ehrlichia canis. Am. J. Vet. Res., 1980; 41: 250–254.
16. **M Nyindo, D L Huxsoll, M Ristic, I Kakoma, J L Brown, C A Carson, E H Stephenson.** Cell-mediated and humoral immune responses of German Shepherd Dogs and Beagles to experimental infection with Ehrlichia canis. Am J Vet Res, 1980; 41(2): 250–4.120.
17. **Harrus S, Kass PH, Klement E, Waner T.** Canine monocytic ehrlichiosis: a retrospective study of 100 cases, and an epidemiological investigation of prognostic indicators for the disease. Vet Rec, 1970; 141(14): 360–3.
18. **Livio Martins Costa Jr 1, Karin Rembeck, Múcio Flávio Barbosa Ribeiro, Pamela Beelitz, Kurt Pfister, Lygia Maria Friche Passos.** Sero-prevalence and risk indicators for canine ehrlichiosis in three rural areas of Brazil. Vet J, 2007; 174(3): 673–6.
19. **Sergio Eduardo Bermúdez Castillero, Carlos Daza, Julio Osorio, Amilcar Hurtado, José Suárez, Ana Santamaria.** Characterization of the First Case of Human Infection by Ehrlichia canis in Panama. Rev Med Panama, 2008; 38(3): 63-68.
20. **Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y. 2006.** Human infection with Ehrlichia canis accompanied by clinical signs in Venezuela. Ann N Y Acad Sci.2006; 1078(1): 110-117.

http://dx.doi.org/10.1196/annals.1374.016. PMid:17114689.

21. **Harrus S. & Waner T.** Diagnosis of canine monocytotropic ehrlichiosis (Ehrlichia canis): an overview. The Veterinary Journal, 2011; 187: 292–296.
22. **Rani P.A.M.A., Irwin P.J., Coleman G.T., Gatne M. & Traub R.J.** A survey of canine tick- borne diseases in India. Parasites & Vectors, 2011; 4: 141.
23. **Sasaki H., Ichikawa Y., Sakata Y., Endo Y., Nishigaki K., Matsumoto K. & Inokuma H.** Molecular survey of Rickettsia, Ehrlichia, and Anaplasma infection of domestic cats in Japan. Ticks and Tick-Borne Diseases, 2012; 3: 308–311.
24. **Adrian P Ybañez 1, Zandro O Perez, Shirleny R Gabotero, Ryan T Yandug, Matsumoto Kotaro, Hisashi Inokuma.** First molecular detection of Ehrlichia canis and Anaplasma platys in ticks from dogs in Cebu, Philippines. Ticks and Tick-Borne Diseases, **2012;** 3: 288–293.
25. **Aktas M., Özübek S., Altay K., Ipek N. D., Balkaya I., Utuk A. E., Kırbas A., Şimsek S. & Dumanlı N.** Molecular detection of tickborne rickettsial and protozoan pathogens in domestic dogs from Turkey. Parasites & Vectors, 2015; 8: 157.
26. **Inpankaew T., Hii S.F., Chimnoi W. & Traub R.J.** Canine vector-borne pathogens in semi-domesticated dogs residing in northern Cambodia. Parasites & Vectors, 2016; 9: 253.
27. **Otranto D., Dantas-Torres F., Weigl S., Latrofa M.S., Stanneck D., Decaprariis D., Capelli G.& Baneth G.** Diagnosis of Hepatozoon canis in young dogs by cytology and PCR. Parasit Vectors, 2011; 4: 55.
28. **Gomes P.V., Mundim M.J.S., Mundim A.V., de Avila D.F., Guimarães E.C. & Cury M.C.** Occurrence of Hepatozoon sp. in dogs in the urban area originating from a municipality in southeastern Brazil. Veterinary Parasitology, 2010; 174: 155–161.
29. **De Miranda R.L., O'Dwyer L.H., De Castro J.R., Metzger B., Rubini A.S., Mundim A.V., Eyal O., Talmi-Frank D., Cury M.C., Baneth G.** Prevalence and molecular characterization of Hepatozoon canis in dogs from urban and rural areas in Southeast Brazil. Research in Veterinary Science, 2014; 97: 325–328.
30. **McQuiston J.H., McCall C.L., Nicholson W.L.** Ehrlichiosis and related infections. J Am Vet Med Assoc, 2003; 223: 1750-1756.
31. **Derakhshandeh N., Sharifiyazdi H., Hasiri M.A.** Molecular detection of Ehrlichia spp. in blood samples of dogs in southern Iran using polymerase chain reaction. Veterinary Research Forum, 2017; 8(4): 347 – 351.
32. **Milanjeet, Singh H., Singh N.K., Singh N.D., Singh C., Rath S.S.** Molecular prevalence and risk factors for the occurrence of canine monocytic ehrlichiosis. Vet Med, 2014; 59(3): 129-136.
33. **Maazi N., Malmasi A., Shayan P., Nassiri S. M., Salehi T. Z., and Fard M. S.** Molecular and serological detection of Ehrlichia canis in naturally exposed dogs in Iran: An analysis on associated risk factors. Rev Bras Parasitol Vet, 2013; 23: 16-22.
34. **Solano-Gallego L., Trotta M., Razia L., Furlanello T., and Caldin M.** Molecular survey of Ehrlichia canis and Anaplasma phagocytophilum from blood of dogs in Italy. Ann N Y Acad Sci, 2006; 1078: 515-518.
35. **Allison, R.W., Little, S.E.** Diagnosis of rickettsial diseases in dogs and cats. Vet. Clin. Pathol, 2013; 42: 127–144.
36. **NakaghiI A.C., MachadoI R.Z., Costa M.T., André M.R., and Baldani C.D.** Canine ehrlichiosis: clinical, haematological**,** Serological and molecular aspects. Ciência Rural, 2008; 38: 766‑770.
37. **Selim A., Ahmed S., and Galila E.** Epidemiological and molecular diagnosis of Ehrlichia canis infection among dogs. Benha Veterinary Medical Journal, 2019; 37: 169-171.
38. **Selim A., Abdelhady A. and Alahadeb J.** Prevalence and first molecular characterization of Ehrlichia canis in Egyptian dogs. Pak Vet J, 2020. http://dx.doi.org/10.29261/pakvetj/2020.061.
39. **Vaden S.L., Knoll J.S., Smith F.W.K., Tilley L.P.** Blackwell's Five-minute Veterinary Consult Laboratory Tests and Diagnostic Procedures: Canine and Feline. Wiley-Blackwell, 2009.
40. **Gal A., Loeb E., Yisaschar-Mekuzas Y., and Baneth G.** Detection of Ehrlichia canis by PCR in different tissues obtained during necropsy from dogs surveyed for naturally occurring canine monocytic ehrlichiosis. The Veterinary Journal, 2008; 175: 212–217.
41. **Inokuma H., Beppu T., Okuda M., Shimada Y., and Sakata Y.** Epidemiological survey of Anaplasma platys and Ehrlichia canis using ticks collected from dogs in Japan. Veterinary Parasitology, 2003; 115: 343–348.
42. **Inokuma H., Parola P., Raoult D., and Brouqui P.** Molecular survey of Ehrlichia infection in ticks from animals in Yamaguchi Prefecture, Japan. Veterinary Parasitology, 2001; 99: 335– 339.
43. **Neer T.M., Breitschwerdt E.B., Greene R.T.,and Lappin M.R.** Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. American College of Veterinary Internal Medicine. J Vet Intern Med, 2002; 16(3): 309–15.
44. **Botros B.A.M., Elmolla M.S., Salib A. W., Calamai C.A., Dasch G.A. and Arthur R.R.** Canine ehrlichiosis in Egypt: Sere-epidemiological survey. Onderstepoort Journal of Veterinary Research, 1995; 62: 41-43.
45. **Stich R.W., Blagburn B.L., Bowman D.D., Carpenter C., Cortinas M.R., Ewing S.A., Foley D., Foley J.E., Gaff H., Hickling G.J., Lash R.R., Little S.E., Lund C., Lund R., Mather, T.N., Needham G.R., Nicholson W.L., Sharp J.,**

Varela-Stokes A., Wang D. Quantitative factors proposed to influence the prevalence of canine tick-borne disease agents in the United States. Parasites Vectors, 2014; 7: 417.

46. **Costa J.O., Batista J.A., Jr. Silva M., Guimaraes M.P.** Ehrlichia canis infection in dog in Belo Horizonte, Brazil. Arq Esc Vet UFMG, 1973; 25: 199-200.
47. **Khazeni A., Telmadarraiy Z., Oshaghi M.A., Mehdi Mohebali M., Zarei Z., Abtahi S.M. 2013.** Molecular detection of Ehrlichia canis in ticks population collected on dogs in Meshkin- Shahr, Ardebil province, Iran. J Biomed Sci Eng, 2013; 6: 1-5.
48. **Khazeni A., Telmadarraiy Z., Oshaghi M.A., Mehdi Mohebali M., Zarei Z., Abtahi S.M. 2013.** Molecular detection of Ehrlichia canis in ticks population collected on dogs in Meshkin-Shahr, Ardebil province, Iran. J Biomed Sci Eng, 2013; 6: 1-5.
49. **Salem, N. Y., Rakha G. H., and Baraka T. A.** Naturally occurring ehrlichiosis in Egyptian dogs, Iranian Journal of Veterinary Research, Shiraz University, IJVR, 2014; 15: 54-57.