Retrospective Study of Tick-Borne Pathogens and Observation of *Ehrlichia ewingii* / *Anaplasma phagocytophilum* and Hemotropic *Mycoplasma* spp. in Dogs’ Blood Films

Maryam Rassouli¹, ² *, Ghazaleh Aghazamani³

¹Pathobiology Department, Shahmirzad School of Veterinary Medicine, Semnan University, Semnan, Iran
²Pathobiology Department, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran
³Shahmirzad School of Veterinary Medicine, Semnan University, Semnan, Iran

Email address:
Mrdvmp@yahoo.com (M. Rassouli), Mrvpar@semnan.ac.ir (M. Rassouli), Ghazale.zamani3@yahoo.com (G. Aghazamani)

To cite this article:

Abstract: *Ehrlichia ewingii*, *Anaplasma phagocytophilum* and hemotropic *Mycoplasma* spp. are three bacteria which can infect different dog’s blood cells. All of these three pathogens can be transmitted by different ticks and some reservoir hosts also play a role in their transmission. Although *E. ewingii* and *A. phagocytophilum* infect granulocytes and neutrophils of their hosts, respectively, hemotropic *Mycoplasma* spp. can infect reticulocytes. In this research, the previously taken dogs’ blood films were collected randomly from different veterinary hospitals of Tehran, the capital of Iran. The blood films were reinvestigated for arthropod-borne diseases. Surprisingly, *E. ewingii* / *A. phagocytophilum* morulae and hemotropic *Mycoplasma* spp. were observed in 18% and 37.7% of samples, respectively. None of these pathogens have been reported in Iran. In addition to the new report, the changes which have been made by these pathogens, their similarities, differences and zoonotic importance are discussed.

Keywords: *Ehrlichia ewingii*, *Anaplasma phagocytophilum*, *Mycoplasma*, Dog, Iran

1. Introduction

Ehrlichiosis is caused by several bacterial species in the genus *Ehrlichia*, pronounced (err-lick-ee-uh) [1], family Anaplasmataceae, order Rickettsiales. They are small, pleomorphic Gram-negative bacteria found in circulating leukocyte or platelets of susceptible mammalian hosts. *Ehrlichia* spp. are tick-borne, obligatory intracellular pathogens. These organisms are often observed in compact colonies or phagosomal inclusions which are named morula [2]. Canine ehrlichiosis is caused by *Ehrlichia canis*. This disease is distributed worldwide and its primary vectors are brown dog tick (*Rhipicephalus sanguineus*) and *Dermacentor variabilis*. This species of *Ehrlichia* commonly infect mononuclear white blood cells [1]. In addition to *E. canis*, *E. chafeensis*, the agent of human monocytic monocytotropic ehrlichiosis (HME) can infect dogs’ mononuclear leukocytes. *E. ewingii* causes canine granulocytic ehrlichiosis, this disease is reported in the US and its potential vector is *Amblyomma americanum*. Granulocytes are the most infected cells [1]. *Anaplasma phagocytophilum* is another tick-borne pathogen of family Anaplasmataceae which can be transmitted by different species of *Ixodes* [3]. *A. phagocytophilum* infection was first described in Human. It also can infect horses, ruminants, dogs and cats. Macaques and baboons were infected experimentally [4]. Small mammals can be reservoir of *A. phagocytophilum* [3].

Hemotropic mycoplasmosis (formely, hemobartonellosis) is caused by wall-less Gram-negative bacterial organisms, *Mycoplasma* spp., class Mollicutes. These hemotropic *Mycoplasma* spp. attach and grow on the surface of infected reticulocytes [5]. *Mycoplasma haemocanis* (formely, *Hemobartonella canis*) and *Candidatus Mycoplasma haematoparvum* cause hemotropic mycoplasmosis in dogs. *M. haemocanis* was experimentally transmitted by *R.
A. phagocytophilum, E. ewingii, bitches, 28 studs) which were referred to different veterinary pathogens. The observed abnormalities were recorded and analyzed because they had not mostly been recorded in the case histories.

2. Materials and Methods

61 blood films were randomly collected from dogs (33 bitches, 28 studs) which were referred to different veterinary hospitals of Tehran, the capital of Iran. Dogs’ age and breed were recorded in a few cases. Previously taken blood films were observed again for hemotropic arthropod-borne pathogens. The observed abnormalities were recorded and compared with previous diagnosis. The newly recorded diseases were analyzed among studs and bitches by chi-square test, SPSS software. The breeds and ages were not analyzed because there had been no access to the previously referred dogs’ whole blood samples and the E. ewingii / A. phagocytophilum could not be differentiate because the dogs’ sera were not available in the referred veterinary hospitals. There was no significant difference between infected studs and bitches (P>0.05).

In Diff Quick of group A, in 11 samples mature neutrophils were decreased but band cells increased, the other leukocytes were normal in counting. In 2 cases were leukopenic. In 2 cases lymphocytosis was observed in addition to mature neutropenia and band cell increasing. In band cell increasing samples, the band cells of 3 samples was 20%-30%, 9 samples were 10-20% and 1 sample 5-10%. There were toxic changes in addition to left shift in neutrophils. Polychromatophilic macrocytes, hypochromic reticulocytes and anisocytosis were also observed in all samples (Figures 1, 3).

In group B, all of the E. ewingii / A. phagocytophilum morulae were observed in neutrophil cytoplasm. In Diff Quick, 2 samples were leukopenic with hypocromatic reticulocytes, demonstrated as discocytes. Poikilocytosis include: discocyte, codocyte, echinocyte, Elliptoctype, echinoelliptocyte and shistocyte. In 1 sample lymphopenia with monocytes was observed. Reactive lymphocytes were also observed in this group (Figures 2, 3).

In group AB: in Diff Quick, 5 samples were leukopenic, 3 cases had mature neutropenia and band cell increasing (1 sample 30-40% and the rest 2 cases 10-20% of the counted leukocytes were band cells). In 1 case in addition to mature neutropenia and band cell increasing, Lymphopenia, monocytosis and reactive lymphocytosis could be observed. Reticulocyte changes include: hypocromasias, anisocytosis and poikilocytosis.

In all leukopenic samples, only neutrophils could be counted in low numbers.
Figure. 1. a: hemotropic Mycoplasma sp. closed to reticulocyte membrane, black arrow. Note the discocytes, 300X, stained by Giemsa. b: hemotropic Mycoplasma sp. closed to reticulocyte membrane, black arrow. Note the discocytes and hyperchromatic macrocytes, 200X, stained by Giemsa. c: doublet of hemotropic Mycoplasma sp., black arrow, 400X, stained by Giemsa. d: hemotropic Mycoplasma sp. closed to two reticulocyte membranes, black arrows, 500X, stained by Giemsa.

Figure. 2. a: Ehrlichia ewingii/Anaplasma phagocytophilum inclusion in a neutrophil cytoplasm, black arrow, 300X, stained by Giemsa. b: Ehrlichia ewingii/Anaplasma phagocytophilum inclusion in a neutrophil cytoplasm, black arrow, 400X, stained by Giemsa. c: Basophilic Ehrlichia ewingii/Anaplasma phagocytophilum inclusions on neutrophil nuclei, thin black arrows and hemotropic Mycoplasma sp. closed to reticulocyte membrane, thick black arrow. Note the discocytes and rouleaux. 400X, stained by Giemsa. d: Ehrlichia ewingii/Anaplasma phagocytophilum inclusion in a band cell cytoplasm, black arrow. 500X, stained by Giemsa. e: Ehrlichia ewingii/Anaplasma phagocytophilum inclusion in a neutrophil cytoplasm, 500X, stained by Giemsa.

Figure. 3. a: poikilocytosis, note the echinocytic, black arrow, 400X, stained by Giemsa. b: hypochromasia, poikilocytosis and anisocytosis. Note the cedocytes, black arrow, 200X, stained by Giemsa. c: pancytopenia and hypochromasia, 40X, stained by Giemsa.

4. Discussion

According to the research, the most important problem among the samples was history taking. Most of them were incomplete. The age and breed of dogs were rarely recorded. In spite of finding some tick-borne diseases, tick infestation history was recorded in only 6 samples. None of these observed abnormalities in the blood films were recorded as a “diagnosed disease” and most of the referred dogs’ diseases had been diagnosed as viral infections. The concurrent viral infections cannot be rejected but these tick-borne infections (E. ewingii/A. phagocytophilum and hemotropic Mycoplasma spp.) may make the infected dogs susceptible to other infectious diseases such as distemper.

Therefore, it can be concluded that blood films were not used for diagnosis and just taken as a routine procedure from referred dogs.

E. ewingii was endemic in southeastern and south-central of the United States and 26% of shelter dogs were seropositive in endemic areas of the U. S [3, 11]. E. ewingii DNA was detected in Cameroon and Brazil [26, 27]. A. phagocytophilum is distributed worldwide. In the United States, A. phagocytophilum is mostly in western and northern Midwestern states [15, 23, 28]. The range of the seroprevalence in infected dogs can vary widely; in the Europe, the A. phagocytophilum seroprevalence may vary from 5% to 70.5% [29-37] and in the United States was from 0.0% to 67.4% [23, 38-40]. In Canada, there was low seroprevalence of A. phagocytophilum among dogs which were tested in different provinces (0.09-0.9%) [3, 41]. A. phagocytophilum DNA was also detected in Europe and North Africa [3].

Canine hemotropic mycoplasmosis was reported in Europe, especially in its Mediterranean countries [42]. In France 3.3% of examined blood samples were infected with M. haemocanis and 9.6% with Candidatus M. haematoparvum [43] and high prevalence of this disease was reported among dogs in Africa [44].

None of these diseases has been reported in Iran, so; the importance of our findings is to prove that E. ewingii/A. phagocytophilum and hemotropic Mycoplasma spp. infections are present among dogs in Iran, therefore; further researches are needed to understand the true prevalence, the importance, species diagnosis and the potential vectors of these diseases. Seroprevalence of E. canis has been reported among dogs in different parts of Iran [45-47]. There is a cross-reactivity between E. ewingii and E. canis in some serological methods [3]. Therefore some of the reported seropositive samples might be related to E. ewingii exposure, but there is not any cross-reactivity between E. ewingii and A. phagocytophilum [3] and serological tests can well differentiate these infections from each other. In this case, because of retrospective study, the access to referred dogs sera was impossible. In this research 23 samples (37.7%) (25.5-50%; 95% confidence interval “CI”) were positive in Mycoplasma spp. and 11 samples (18%) (8.4-27.6%; 95% CI) were positive in E. ewingii/A. phagocytophilum which
are relatively high. One of the results of the disease unidentified is, no treatment of infected dog will occur and consequently high prevalence of the infection among more ticks and more dogs will be observed.

_Amblyomma americanum_ is the main vector of _E. ewingii_ and white-tail deer ( _Odocoileus virginianus_ ) serve as a reservoir for _E. ewingii_ in the southern parts of the U.S [48-50]. _E. ewingii_ DNA was detected in _D. variabilis_ and _Rh. sanguineus_ [14, 51-54]. These species and may be some other ticks are more important in other countries such as Brazil and Cameroon where _E. ewingii_ has been detected [26, 27]. Once ticks are infected by _E. ewingii_, they remain infected throughout their life (transstadial transmission) [14, 55]. _A. phagocytophilum_ can be transmitted by members of the _Ixodes persulcatus_ complex (e.g., _I. scapularis, I. pacificus, I. ricinus_ and _I. persulcatus_) [3, 56]. A minimum required feeding time for _Ixodes_ spp. which can transmit _A. phagocytophilum_ to susceptible mammalian hosts is 24 to 48 hours [57-59]. _A. phagocytophilum_ is able to reside in the salivary glands of unfed _Ixodes_ spp. ticks [60]. _R. sanguineus_ serves as a vector and reservoir of _M. haemocanis_ because of transstadial and transovarial transmission of the organism [3]. Unfortunately, there is no information of vectors and reservoirs of above pathogens in Iran. _A. americanum_ has not been reported in Iran thus, the _E. ewingii_ vectors can be the other tick genera. It is also predicted that because of the mean temperature increase in Iran, ticks can be active in more months of the year and thus tick-borne diseases will increase. Therefore, tick control is necessary for pets and farm animals.

Observable changes in blood films were recorded in dogs infected with _E. ewingii, A. phagocytophilum_ and _M. haemocanis_ and listed as below; in _E. ewingii_ infection, the most important abnormality is thrombocytopenia but platelet counts are not helpful for diagnosis [9, 10, 12, 14]. Mild anemia and reactive lymphocytosis are observed [10, 11]. Biochemical abnormalities are mild and nonspecific in this disease [3]. Neutrophilic inflammation causes polyarthritis [10], _E. ewingii_ morulae can also be detected in CSF fluid, joint fluid, prostatic fluid or other body fluids [9, 10, 12]. Another problem which is caused by _E. ewingii_ is the anemia is regenerative so hematological abnormalities are observed. Monocytopenia, polychromatophilic macrocytes were observed, so this is another reason for chronic forms of these diseases in Iran because of the unidentification.

Neutrophilia, monocytosis and lymphopenia can occur in a response to endogenous or exogenous glucocorticoids in dogs like other animals [67-70]. Potential causes of increased endogenous releasing of glucocorticoids include fever, pain, stress, intense exercise and hyperadrenocorticism [69, 71]. Left shift is often associated with inflammatory conditions. Bacterial infections, among other infectious diseases are more important for left shift occurrence [72, 73]. Glucocorticoids increase lymphocyte sequestration and apoptosis [74-76], some secreted cytokines in bacterial and viral infections also cause lymphopenia [77-79]. Reactive lymphocytes are antigen stimulated lymphocytes which their size and their cytoplasmic basophilia were increased [73]. In this case all of the above abnormalities were observed and mentioned in details in the result section but leukopenia might occur due to chronic _E. ewingii/A. phagocytophilum_ or other concurrent diagnosed diseases like distemper or parvovirus infection! It is also unclear if _E. ewingii/A. phagocytophilum_ and hemotropic _Mycoplasma_ spp. infections can make dogs susceptible to other infections. This question and other ambiguous aspects of these tick-borne diseases must be answered by further researches.

No breed predispositions have been reported in _E. ewingii, A. phagocytophilum_ and _M. haemocanis_ infection [15, 16, 30]. There were some reports of coinfection of _A. phagocytophilum_ with _Borreliia burgdorferi_ in different hosts [80], _A. phagocytophilum_ infected dogs in the US coincided with other _Anaplasma_ spp., _Babesia canis, Babesia vinsonii, E. canis, Rickettsia rickettsia _ [3, 81-83]. In this research only coinfection of _E. ewingii/A. phagocytophilum_ with hemotropic _Mycoplasma_ spp. was observed.

The mortality has not been reported in order to _E. ewingii_ or _A. phagocytophilum_ infection among dogs, thus these diseases are mild or in apparent. _E. ewingii_ infection may clear spontaneously within weeks or several months [84]. Two deaths of puppies were reported due to hemotropic mycoplasmosis [3], _M. haemocanis_ could be successfully transmitted congenitally and orally [3, 85] in experimental studies. _M. haemocanis_ can cause severe life-threatening anemia in splenectomized dogs [3]. According to the results, these diseases have not been diagnosed in Iran, so their mortality rate cannot be estimated.

There are some reports of _E. ewingii_ in immunocompromised human [24, 86-88], but there is no report of dog-to-human transmission [3]. _A. phagocytophilum_ in human is self-limiting such as dogs [3], but pet animals have an important role in epidemiological aspects of _A. phagocytophilum_ in an area [25, 89]. _E. ewingii_ and _A. phagocytophilum_ infections are also important in sheepdogs because both can also infect small ruminants [90, 91]. _Mycoplasma haemofelis_ was detected in an immunocompromised man from Brazil [92]. This case and
some similar cases in human [93, 94] suggest vector, bite, or scratch transmission, or handling blood from animals may transmit the infection into human, especially in immunocompromised individuals [3].

*E. ewingii* can be treated rapidly by suitable antimicrobial therapy. Doxycycline is a choice drug but other tetracycline can also be used [95]. Supportive treatment is necessary in polyarthritis cases [3]. *A. phagocytophilum* responds to doxycycline, rifampin and levofloxacin. Other tetracycline derivatives are also useful [96]. Canine hemotropic mycoplasmosis is never treated completely and after treatment latent infection is still in infected dogs. This disease can be limited by orally administered tetracyclines. Blood transfusion is necessary when the anemia is severe [3].

### 5. Conclusion

*E. ewingii* and *A. phagocytophilum* infection make some similar symptoms in infected dogs and their morulae cannot be differentiated in the blood films [3, 21], but fortunately both bacteria are susceptible to tetracyclines [95]. It is recommended that if the morula was observed in the infected dog’s blood film, treatment must start immediately either the infectious agent is *E. ewingii* or *A. phagocytophilum* but the laboratory should record the result as *E. ewingii/A. phagocytophilum*. Afterward, if it is necessary, the serum is tested by serological techniques. Greene (2012) also mentioned that differential diagnosis between *E. ewingii* and *A. phagocytophilum* is usually done for zoonotic concern (for the owner or academic importance) [3]. In developing countries such as Iran because all of the veterinary laboratories have not been equipped for serological and molecular tests, the differential diagnosis between *E. ewingii* and *A. phagocytophilum* may get in trouble. In this research the accurate diagnosis of the reported infections didn’t occur due to retrospective study limitations which were mentioned above but further researches to complete our findings are necessary.

### Acknowledgments

Special thanks to Dr. Fathipour for his help and there is no competing of financial interests exists.

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Amblyomma americanum
phagocytophila
scapularis
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