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Research Article

Molecular Prevalence, Hematological Biomarker, Associated Risk Factors and Chemotherapeutic Trials of Ehrlichiosis in Dogs in Pakistan

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Abstract: The study was a prospective trial, planned to determine the molecular epidemiology of ehrlichiosis in dogs through blood smear microscopy and Polymerase chain reaction (PCR). A total of 384 cephalic blood samples were collected from domestic (n=288) and stray dogs (n=96) belonging to varied demographics. Molecular detection of *Ehrlichia* spp. was conducted through PCR by targeting 16S rRNA gene using the genus specific primers. Final logistic regression analysis revealed that previous history of tick infestation and housing hygiene were significant (P<0.001) risk factors associated with molecular prevalence of canine Ehrlichiosis. The animals of Group-1 (n=7) received only Minocycline at the rate of 12 mg per kg PO 21 days. The dogs of Group-2 (n=7) received Minocycline at the rate of 12 mg per kg PO with Imidocarb dipropionate at the dose rate of 6.6 mg per kg once. Group-3 (n=7) received Ciprofloxacin 10 mg per kg PO only. Group 4 (n=7) received Ciprofloxacin 10 mg per kg along with Prednisolone 1 mg for 8 days. Success of treatment was evaluated based on PCR tests. The results of the treatment trials revealed 71.42%, 85.71%, 57.14% and 71.42 % recovery rate for Group 1, Group 2, Group 3 and Group 4, respectively. The treatment trial concluded that minocycline along with Imidocarb dipropionate produced highest recovery rates.

Keywords: *Ciprofloxacin, Dogs, Ehrlichiosis, Minocycline, Polymerase chain reaction, PCR*

Pakistan'da Köpeklerde Ehrlichiosis'in Moleküler Prevalansı, Hematolojik Biyobelirteçler, İlgili Risk Faktörleri ve Kemoterapötik Denemeler

Öz: Bu çalışma, kan froti örneklerinin mikroskobik analizi ve Polimeraz zincir reaksiyonu (PCR) yoluyla köpeklerde ehrlichiosis'in moleküler prevalansının belirlenmesi için yapılan prospektif bir çalışmaydı. PCR'de 16S rRNA genini hedefleyen cins-spesifik primerler kullanılarak %9.63 (37/384) oranında genel bir yaygınlık gözlemlendi. Toplam eritrosit sayısı (TEC), Eritrosit sedimentasyon hızı (ESR), Hemoglobin (Hb) düzeyi ve Trombosit sayısı gibi hematolojik biyobelirteçler, hastalıklı bireylerde önemli ölçüde azaldı. Kene istilası öyküsünün varlığı ve kötü barınak hijyeni, hastalıkla önemli ölçüde ilişkili risk faktörleriydi. Enfekte hayvanlar arasında şu şekilde bir kemoterapi denemesi yapıldı; Grup-1 (n=7)'e, Minosiklin uygulandı (21 gün boyunca 12 mg/kg PO); Grup-2 (n=7)'ye, çalışmanın başlangıcında bir kez tek doz (6.6 mg/kg) halinde İmidokarb dipropionat ile birlikte Minosiklin (21 gün boyunca 12 mg/kg PO) uygulandı, Grup-3 (n=7)'e, Siprofloksasin (21 gün süreyle 10 mg/kg PO) uygulandı, Grup 4 (n=7)'e 8 gün süreyle Siprofloksasin (10 mg/kg) ve Prednisolon (1 mg) uygulandı ve Grup 5 (n=7)'teki hayvanlar Kontrol grubu olarak kabul edildi. Tedavinin başarısı, PCR testlerine göre değerlendirildi ve Grup 1, Grup 2, Grup 3 ve Grup 4 için sırasıyla %71.42, %85.71, %57.14 ve %71.42 iyileşme oranlarını elde edildi. Tedavi denemesi sonucu, Minosiklinin, Imidocarb dipropionate ile birlikte uygulamasının en yüksek iyileşme oranı sağladığı sonucuna varılmıştır.

Anahtar sözcükler: *Siprofloksasin, Köpek, Ehrlichiosis, Minosiklin, Polimeraz zincir reaksiyonu, PCR*

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Introduction

Canine vector-borne diseases (CVBDs) are caused by a diverse range of bacteria, viruses, and eukaryotic parasites that are conveyed by arthropod blood-sucking vectors, mostly ticks and mosquitoes [1]. Several critical variables, including a change in global climatic conditions could explain the exponential spread of arthropod vectors and CVBDs [2]. Ticks have emerged as the most pernicious arthropod vector in several different ecological habitats. Some of these CVBDs present a serious zoonotic threat [3]. Dog population has been incrementally rising in the last couple of decades owing to the fact that this trend of keeping pet dogs has gained greater cultural acceptance [4]. This shift has affected global distribution of CVBDs as well. Climate change, ease of international transportation, and rapid rise in human, canine, and other reservoir animal populations have proven to be key factors in this regard [5]. Climate has a significant impact on the survival and dissemination of arthropod vectors, as well as the dispersion of CVBDs [3].

The *Ehrlichia* species belongs to the family *Anaplasmataceae*, capable of infecting canine, bovine and human hosts. Ehrlichiosis is a disease caused by an obligatory intracellular Gram-negative bacterium that replicates in the host's mononuclear cells [3]. The pathogen is mainly transmitted by so called brown dog tick, *Rhipicephalus sanguineus* [5]. High grade fever, anorexia, dullness, enlarged spleen, pancytopenia and spontaneous hemorrhagic tendencies are the most obvious clinical signs [4]. The incidence of ehrlichiosis is steeply elevated during peak tick infestation seasons i.e., spring and autumn [5]. The ehrlichiosis has the zoonotic potential and believed to be common in both rural and urban areas of tropics. Molecular and serological diagnostic tests have validated the existence of *Ehrlichia* in cats, dogs, wild animals, and humans [4]. A Brazilian study revealed that, *Ehrlichia canis* is the most prevalent species found amongst dogs. The global prevalence for *Anaplasma phagocytophilum*, and *E. canis*, were reported to be 1.6% and 6.3% respectively. The development of tick-borne disease has been linked to age and severity of tick infestation [6].

The clinical diagnosis of Ehrlichiosis in animals is difficult because the clinical signs are ambiguous and the serological assays have limited diagnostic application. The isolation of pathogen is difficult and requires a tissue culture medium for its growth [5]. Cytoplasmic inclusion bodies called morulae could be identified during microscopic examination of blood smears but its limited sensitivity as compared to serological or molecular assays impede widescale implementation [6]. Ehrlichiosis has worldwide distribution. Australia was previously believed to be free from Canine Ehrlichiosis but was recently discovered in its northern and western territories [2]. In Pakistan, 24% prevalence of ehrlichiosis has been previously reported in dogs [7]. Minocycline a new drug that belongs to tetracycline antibiotics group is regarded as the cornerstone for chemotherapy and the drug of choice for treatment of Canine Ehrlichiosis [7]. Previous studies have reported considerable efficacy for both minocycline and imidocarb dipropionate [6]. This combination had proved most effective and successful treatment for resolving clinical disease and pathogen eradication. Minocycline, a close relative of doxycycline, is an obvious choice for *E. canis* as well as other members of *Anaplasmataceae* and *Rickettsiales* families [8]. Minocycline may be a better choice for treating these elusive bacteria than doxycycline because of its high lipophilicity, minimal protein binding, and greater penetration into tissues including the brain [2]. In the present study, authors have endeavored to establish the most efficacious drug against canine ehrlichiosis by firstly identifying positive samples, establishing aggravating factors and compare the efficacy of minocycline with treatment regimens that have been proven effective in prior publications.

Material and Methods

Ethical Consideration

Approval of this study was obtained from the University of Veterinary and Animal Sciences, Lahore, Thesis Committee (Approval no: 8226). This research was a prospective trial, therefore only clinical patients were inducted after informed consent was obtained from their respective caretakers. Research design was in complete compliance with the established guidelines stated in Pakistan's Prevention of Cruelty to Animals Act (1890), Punjab Wildlife Protection, Preservation, Conservation and Management Act (1974).

Research Area

Dogs were sampled in district Sheikhupura of Punjab Province, to estimate the seroprevalence, associated risk factors and relative efficacy of various drug regimens against canine ehrlichiosis. The climate of study area was semi-arid and sampling was performed between March and September of 2021. The GIS map shows different locations from where blood samples were collected *(Fig. 1)*.

Collection of Samples

A total of 384 blood samples of clinically infected dogs (domestic, n=288 and stray, n=96) were selected belonging to different breeds, age and gender from different government as well as private kennels to estimate the prevalence, risk factors and efficacy of different drugs used against canine ehrlichiosis. All the dogs were included in sampling frame.

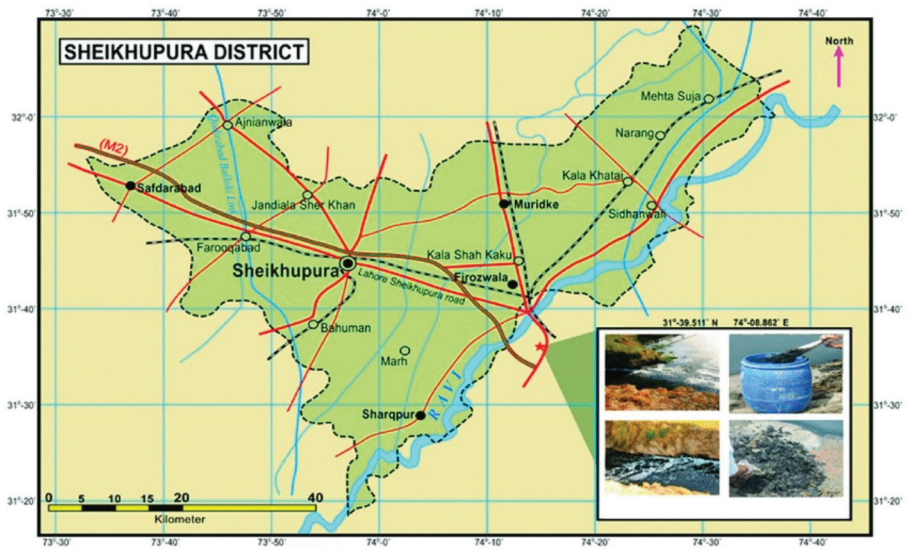

Fig 1. GIS Map showing the study area

The samples were transferred in an ice box containing ice pack and brought to laboratory for further processing.

Inclusion and Exclusion Criteria

Dogs with history of tick infestation, anemia, lymphadenitis, excessive weight loss, fever above 104˚F, spontaneous bleeding disorders and no prior treatment history within last one month were selected for this prospective epidemiological trial. Dogs suffering from any major infectious or non-infectious disease, other than ehrlichiosis were excluded from the study. A questionnaire was used to collect information regarding area, host, breed, gender, age, and number of dogs, extent of veterinary care, health status and periodicity of tick infestation etc.

Methodology

Thin Blood Smears and Phlebotomy

Two methods of sampling were employed for detection of ehrlichiosis from blood. Firstly, thin blood smears (in triplet) were created from an ear vein and air dried on the spot. Secondly, 3 mL blood was taken aseptically from the cephalic vein into EDTA-coated vacutainers (medivac). The samples were delivered to the laboratory where the cold chain was maintained. For each sample animal description, managemental, and environmental determinants were captured on a data collection form [9].

Microscopic Examination of Blood Smears

The blood smears were fixed in a 10% ethanol solution and stained with a Giemsa stain. The stain was wiped away with running tap water after 15 min, and the discolored smears were allowed to dry. The streaks were spotted using a 100x oil immersion lens. The smears were examined for intracytoplasmic inclusion bodies in blood cells that looked similar to the ones observed in case of *Anaplasma* spp.[9]. Detection of such inclusion bodies would support primary animal screening.

Genomic Analysis

The DNA was extracted from the blood samples of dogs by using an Exgene™ (GeneAll®) DNA extraction kit [10]. The sampled blood was prepared for DNA extraction by mixing it with absolute ethanol in 1.5 mL to maintain a final volume of 200 µL. Solution was repeatedly centrifuged and incubated subsequent to addition of 20 µL of Proteinase K, 200 µL of buffer Bl and 200 µL of absolute ethanol respectively. The entirety of the solution was transferred to an SV column and centrifuged at 6000 rpm until all the solution had passed through the membrane and became colorless. The mixture was pipetted to another tube and centrifuged after the addition of 700 µL of buffer TW. Finally, the SV column was transferred to another microcentrifuge tube and 200 µL of buffer AE was added before a final round of centrifugation and incubation.

The DNAs after extraction from the blood samples were taken to purity and concentration measurements with the help of Gel electrophoresis. On average the DNA yield per sample was 40 ng/µL. This showed that the samples had suitable DNA for amplification through PCR. The DNA quantity was assessed by using nano drop [10]. A primer targeting 16S rRNA gene of *Ehrlichia* spp. was utilized. Using the appropriate bioinformatics tool, the forward primer i.e., Ehr-F2: 5-AATAATAATGCTGGTCAAGT ATGGAATCAT-3; and the reverse primer Ehr-R2: 5-AAGCGTGTTCCCATACATCCATAG-3 were used to amplify the 16S rRNA gene [7,11,12]. PCRs were carried out in a final volume of 20 µL with 10 µL of TOP real TM qPCR 2x Pre-Mix, 4 µL of DNA, 2 µL (10 pmol) of each primer, and 2 µL of distilled water. Initial denaturation was conducted at 95°C for 5 min, after which the reaction was cycled 40 times with denaturation at 95°C, annealing at 57°C, and extension at 72°C, each step lasting 30 sec, and the last extension at 72°C lasting 10 min. The amplification of gene was carried out in a thermal cycler

(Scilogex PC1000-G™) according to the guidelines of the manufacturer. The PCR products were seen on a UV illuminator in a 1.5% ethidium bromide-stained agarose gel at 120 volts and 200 amperes following gel electrophoresis with an expected size ranging between 300-400 base pairs [13]. Samples positive for *Ehrlichia canis* were graciously donated by Dr. Muhammad Zia (PhD Scholar) from Department of Parasitology, University of Veterinary and Animal Sciences Lahore. A 100bp DNA ladder (BioShop®, Canada) along with negative controls (PCR mixture without DNA) were amplified during each PCR as well.

Hematological Analysis

Complete blood cell count was carried out using hematology analyzer (BioSystems BTS-350®). A 3 mL sample of blood was taken straight from the cephalic vein into an EDTA vacutainer from 10 positive dogs based on PCR and 10 healthy dogs. Using a hematology analyzer, several hematological parameters such as total erythrocyte count (TEC), total leukocyte count (TLC), hemoglobin (Hb), platelet count, and packed cell volume (PCV) were measured [14].

Chemotherapeutic Clinical Trial

After screening, dogs were divided into five groups; comprising seven dogs in each group. Following treatments were given to each group to determine the efficacy of different drugs against Ehrlichiosis in dogs:

Group-1 (n=7): Minocycline 12 mg/kg PO (21 days); Group-2 (n=7): Minocycline 12 mg/kg PO (21 days) with Imizole (imidocarb dipropionate) 6.6 mg/kg subcutaneously once; Group-3 (n=7): Ciprofloxacin 10 mg/kg PO (21 days); Group 4 (n=7): Ciprofloxacin 10 mg/kg PO with Prednisolone 1 mg (8 days); Group 5 (n=7): Control (non-treated). The efficacy for each drug was determined by percentage of recovery from each drug and consequent improvement in hematological parameters [14].

Statistical Analysis

The data regarding the risk factors were analyzed using logistic regression model. Data regarding comparative therapeutic efficacies during treatment trials was assessed using paired t-Test, keeping level of significance (P<0.05). All the statistical analyses were performed by SPSS version 26.0 (version 26, IBM, Chicago, IL).

Results

Prevalence of Ehrlichiosis

A total of 384 samples were collected and screened for *Ehrlichia* infection by blood smear and molecular examination. The blood smear examination of samples revealed that out of 384 samples, 22 were found positive for inclusion bodies resembling *Ehrlichia* with a positive percentage of 5.73% *(Fig. 2)*.

The PCR analysis of samples revealed an overall prevalence of 9.63% (37/384) in dogs *(Table 1) (Fig. 3)*.

Risk Factors Associated with Ehrlichiosis in Dogs

The relationship of assumed risk factors like breed, sex, age, tick infestation, history of tick infestation, type of acaricide, hygiene condition and acaricide interval were analyzed statistically to find out association with occurrence of disease *(Table 2)*. The risk factors were initially analyzed using the chi-square method and the variables having (P<0.01) were further analyzed using a multivariable logistic regression model *(Table 2)*.

The risk factors like tick infestation, past tick history and house hygiene are considered significant risk factors towards the occurrence of Ehrlichiosis in dogs as the P-value is less than 0.05. These significant risk factors were further analyzed by logistic regression model to find out the association between these factors in causation of Ehrlichiosis in dogs. Based on final regression model, only two risk factors were found potential risk factors towards the incidence of disease (P<0.05). The dogs having history of tick infestation in the past were at 3.103 times more

Table 1. *Overall prevalence of Ehrlichiosis in dogs determined through Blood smear microscopy and PCR test*

Diagnostic Test	Positive	Prevalence (%)
PCR positive	37/384	9.63
Microscopy positive	22/384	5.73

Percentage Prevalence have been calculated by running the same samples from 384 Dogs through both diagnostic tests

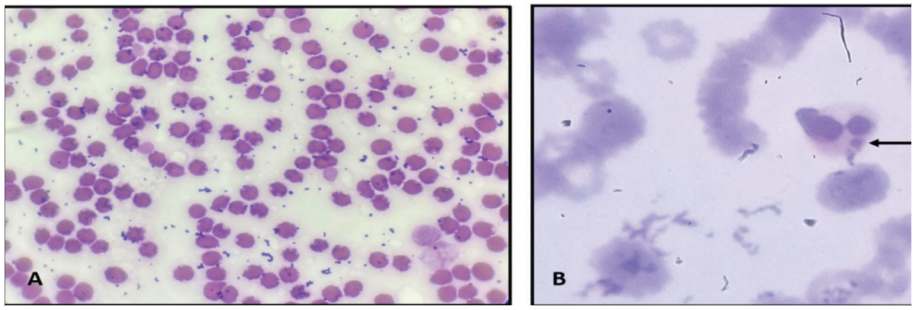

Fig 2. Photomicrographs of a thin blood smear sampled from infected dog: (A) Presence of *Ehrlichia* like intracytoplasmic inclusion bodies in monocytes (Giemsa staining, 200x); (B) An arrow has been used to identify intracytoplasmic morula of *Ehrlichia canis* in a magnified image of dog's monocyte (Giemsa staining, 400x)

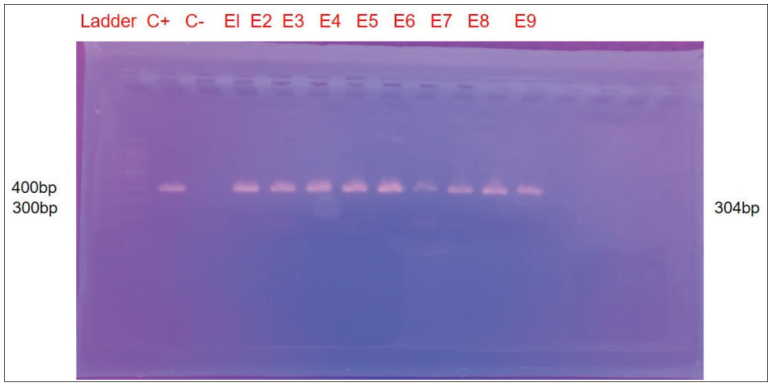

Fig 3. Results for agarose gel electrophoresis of PCR for *Ehrlichia* at 304bp: Lane 1 contains a 100bp DNA ladder (BioShop®, Canada); Lane 2 is Positive Control (C+); Lane 3 is Negative Control (C-); Lane 4 to 12 contain DNA purified from dog samples suspected for Ehrlichiosis and are represented as E1 to E9. Numbers on the left indicate molecular sizes in base pairs.

Table 2. *Summary of risk factors related with Ehrlichiosis in dogs*

Variables	Level of Variable	No. of Samples (n=384)	Positive (%)	Negative	P-Value
Breed	Labrador	45	26 (57.78)	19	0.06
	German Shepherd	135	54 (40.00)	81	
	Bully	115	50 (43.10)	65	
	Non-descript	89	45 (50.56)	44	
Sex	Male	193	91 (47.15)	102	0.64
	Female	191	86 (44.79)	105	
Age	<1 year	244	159 (64.89)	85	0.89
	> 1 year	140	18 (12.85)	122	
Tick Infestation	Absent	265	89 (33.58)	176	<0.001
	Present	119	88 (73.94)	31	
History of tick infestation	Yes	224	126 (56.25)	98	<0.001
	No	160	51 (31.87)	109	
Hygiene condition	Good	147	48 (32.65)	99	<0.001
	Poor	237	129 (54.43)	108	
Type of acaricide	Topical	151	73 (48.34)	78	0.52
	Parenteral	163	76 (46.62)	87	
	Not applied	70	28 (40.00)	42	
Acaricide interval	> 3 months	353	162 (45.89)	191	0.90
	< 3 months	24	12 (50.00)	12	
	Not applied	07	03 (42.85)	04	

Values are represented in terms of positive and negative samples for Ehrlichiosis. A P<0.05 indicated statistical significance amongst corresponding variables

risk at acquiring diseases as compared to animals having no previous tick history. The P-value is also less than 0.05 and it is considered as true risk factor. However, the dogs existing in poor hygienic conditions have 3.095 times more chances of disease occurrence as compared to the dogs living in good hygienic measures and P-value is also less than 0.05 *(Table 3)*.

Effects on Hematological Parameters at Different Time Periods During Chemotherapy

To determine the influence of *Ehrlichia* on various haematological parameters, a comparative hematological analysis was done on *Ehrlichia* positive and healthy animals. The obtained findings were evaluated using an independent T-test, and it was discovered that in dogs infected with *Ehrlichia*, there was a significant (P<0.05) drop in Total Erythrocyte Count (TEC), Erythrocyte Sedimentation Rate (ESR), Hemoglobin (Hb) level and Platelet count. The comparative hematological study revealed a significant (P<0.05) decrease of TLC in acutely diseased dogs compared to the healthy ones *(Fig. 4)*.

Therapeutic Trials Against Canine Ehrlichiosis

The efficacy of three antibiotics was measured on the basis of disappearance of clinical signs and hematological parameters at 7, 14 and 21 days after initiation of therapy *(Table 4)*.

Table 3. *Risk factors included in final logistic regression model*						
Variables	**Variable Levels**	**Odd Ratio**	**95% C.I.**		**S. E**	**P-Value**
			Lower	**Upper**		
Tick Infestation	Absent	1	0.803 - 6.583		0.537	0.121
	Present	2.299				
History of tick infestation	No	1	1.035 - 9.306		0.560	0.043
	Yes	3.103				
Hygiene condition	Good	1	1.102 - 8.694		0.527	0.032
	Poor	3.095				
Relative probability for Ehrlichiosis in presence or absence of a variable has been presented in the table. A P<0.05 indicated statistical significance						

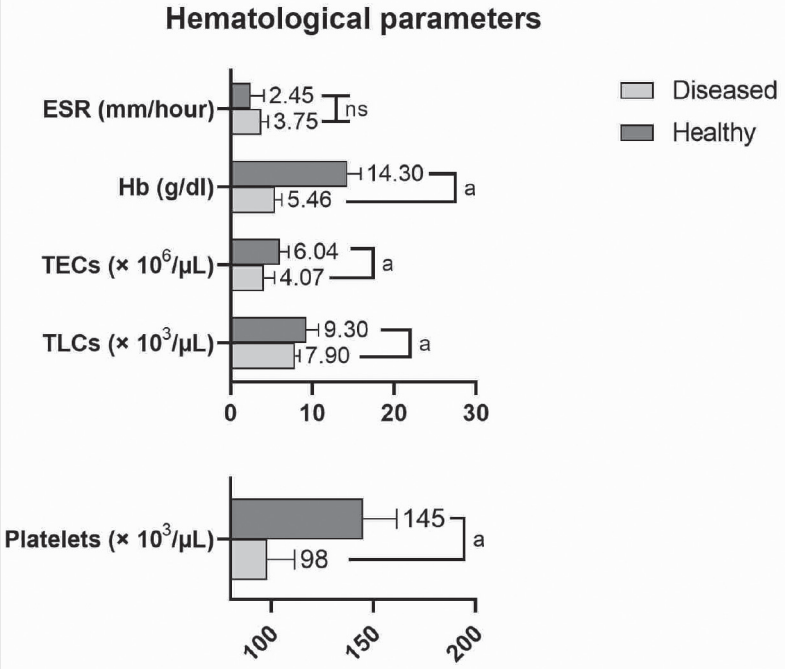

Fig 4. Interleaved Bars graph illustrating a comparison of Erythrocyte sedimentation rates (ESR), Hemoglobin (Hb), Total Erythrocyte counts (TECs), Total Leucocyte counts (TLCs) and Platelets between diseased and healthy dogs. Superscript i.e., (a) represents the statistical significance whereby (P<0.05) while (ns) denotes non-significance amongst variables

Table 4. *Therapeutic trials of different drugs against Canine Ehrlichiosis*					
Days	**Success of Treatment Trial**				
	Group 1 Minocycline (N=7)	**Group 2 Minocycline + Imidocarb Dipropionate (N=7)**	**Group 3 Ciprofloxacin (N=7)**	**Group 4 Ciprofloxacin + Prednisolone (N=7)**	**Group 5 Control Positive (N=7)**
7 days	3/7	4/7	3/7	3/7	0/7
14 days	4/7	5/7	4/7	4/7	0/7
21 days	5/7	6/7	4/7	5/7	0/7

Minocycline alone performed adequately well and there was nominal statistical difference between the treated groups. However, the treatment trial concluded that minocycline along with Imidocarb dipropionate produced highest recovery rates.

Discussion

Tick-borne infections in dogs are becoming more common across the world, making them crucial for small animal practitioners and public health [7]. Ehrlichiosis in dogs is found and reported all over the world [2]. Variety of factors influences the occurrence of disease including host age, vector distribution, habitat, dog survival, climatic conditions and management approaches [5]. The molecular prevalence of tick-borne illnesses in Pakistan like babesiosis has been documented but limited work has been done on Canine Ehrlichiosis [7]. Pérez-Macchi et al.[15] illustrated the prevalence of Ehrlichiosis in Uruguay amongst dogs using polymerase chain reaction as 10.41%.

While an Argentinian study reported the prevalence of 6.7% [16]. Both these findings were corroborated by present outcomes. However, a prior study conducted in three districts of Punjab, Pakistan reported a relatively higher prevalence (28%) of Ehrlichiosis in dogs [7]. A higher rate of prevalence has been reported in India as well [17]. Whereas a much lower prevalence (2%) was reported in Malaysia [18].

In a Nigerian study where light microscopy and polymerase chain reaction were employed in a similar fashion as in the current one, prevalence of 10.25% of this disease was reported, which was much higher than present findings [19]. Area, weather climatic differences in various regions, test methods, vegetation cover prevalent tick species as well as animal husbandry and agronomic practices have been hypothesized to contribute towards variable disease occurrence [5]. Similar to past findings, authors could not validate breed and sex as disease determinants. Researchers have already elucidated that environmental, ecological, and social variable, rather than immunological characteristics, may pose a significant risk in disease development [20-22]. However, authors observed a sharp disparity of infection rates amongst genders as fewer cases of disease in bitches were reported. Whereas, infection rate in our present study were higher in younger animals (<1 year) than the adult ones (>1 year), which contradicted prior findings [15].

The frequency of tick-borne infections in this study were closely related to the extent of tick infestation. Similar studies in the past have concluded that dogs infested with ticks were 3.3 times more at risk to disease as compared to non-tick infested ones [23,24]. Pakistan is located in the tropical and subtropical regions of the world with an unrelenting and humid climate [20]. It makes it quite suitable for tick proliferation and sustenance. Dogs without proper preventive measures have a greater risk of tick infestation [7]. Authors detected that in dogs infected with *Ehrlichia,* there was a significant ($P<0.05$) drop in TEC, ESR, Hb level and MCHC. These findings are important indicators of anemia in diseased animals [4,25]. Previously, it has been reported that dogs suffering from Ehrlichiosis experience anemia and thrombocytopenia [26]. Acute cases ehrlichiosis in dogs were also presented with spontaneous incidence of lowered Hb, TEC, and platelet counts leading to underlying blood coagulopathy [27].

The molecular methods utilized in this investigation were demonstrated to be very sensitive when compared to blood smear microscopic examinations, a fact that has now been reaffirmed by several epidemiological surveys [28]. Examining stained blood smears is less sensitive and requires technical expertise [10]. It is frequently unsatisfactory because the pathogen is either missing or present in very low levels. Intermittent low parasitemia is a characteristic of persistent infection [10]. Nevertheless, during acute infection, blood smear examination remains the easiest and most accessible diagnostic test for clinicians with reasonably sensitivity [9]. Molecular and serological approaches are better at detecting chronic and subclinical illnesses, and they are mostly useful for epidemiological studies. Despite repeated efforts to improve PCR screening, a negative result should be regarded with care due to the cyclic nature of disease [9].

Therapeutic doses of tetracyclines such as doxycycline have traditionally been used against Canine Monocytic Ehrlichiosis (CME) [26]. Several authors have corroborated their efficacy to inhibit binding of bacterial ribosomes with aminoacyl-tRNA in both experimental as well as natural settings [14,25,29,30]. Earlier reports have suggested a minimum inhibition concentration (MIC) of 0.03 mg/ml for doxycycline [31,32] establishing it as a "gold standard" broad spectrum drug for the treatment of Canine Ehrlichiosis. However, several recent studies have deduced that this presumed efficacy was highly subjective and vastly dependent upon several factors namely dosing regimens, degree of infectious load, host's immunological status, sampling methodology and the sensitivity of assays employed for detection [14,25,29,30]. Moreover, risk of doxycycline resistance in *E. canis* owing to its wide-spread usage in endemic areas could not be underplayed either [33,34]. Additionally, presumed side-effects such as diarrhea, anorexia, vomiting, and elevated hepatic enzymes associated with its prolonged usage have incentivized researchers to investigate clinical efficacy of mino-cycline [25,30]. Being a pharmacological relative of doxycycline, it was an obvious choice for *E. canis* treatment, as well as other members of *Anaplasmataceae* and *Rickettsiaceae* families [25,35]. Minocycline has been found efficacious against CME in prior studies [25,35]. Nevertheless, risk of re-infection has been associated with all tetracyclines, including Minocycline. Therefore, prior publications have suggested studies to investigate its efficacy in combination with drugs such as Imidocarb dipropionate and Metronidazole to counter the possibility of remission [26].

Considering the cyclical nature of Ehrlichiosis, Imidocarb dipropionate has always been considered a viable option for treatment of clinical remissions of CME [36]. A research group at American College of Veterinary Internal Medicine had even suggested it to be a second line of treatment in CME [37]. However, studies have demonstrated its immense capability in providing hematological recovery amongst the most perniciously acute cases as well [26,37]. In a more recent publication, authors have proposed a combination of Minocycline and Imidocarb dipropionate to be the most effective and successful treatment for resolving the clinical manifestation of CME [25]. Authors have been

able to corroborate these prior guidelines in present settings.

Comparable studies have reported Rifampicin, a DNA-dependent RNA polymerase inhibitor to possess similar efficacy to that of doxycycline against *E. canis* [29] but an inability to clear *Ehrlichia chaffeensis* amongst human hosts has rendered it obsolete. Similarly, Enrofloxacin, a DNA gyrase inhibitor has been diligently researched for its efficacy against *E. canis* infection [38]. Though initially found to be quite promising, it failed to provide hematological recovery or clearing of acute experimental *E. canis* infection [26]. The potential for emergence of enrofloxacin resistant *Ehrlichia* spp. has been alluded to, by several researchers as well [39,40]. However, the efficacy of fluoroquinolones for the mitigation of severe aplastic pancytopenia in dogs cannot be disregarded either [26]. Consequently, drugs such as ciprofloxacin have also been used or proposed for treatment of CME due to their immense therapeutic potential and their efficacy in mitigating *E. canis*-associated aplastic pancytopenia [26,40]. Antibiotic usage in a dog suffering from Ehrlichiosis are heavily predicated upon selective intestinal decontamination, negligible effect on platelet function and minimal toxicity [29]. Most researchers have observed greater efficacies in cases where either a combination of different classes of drugs were used or adjuvants were administered to mitigate drug related side-effects.

The findings of this study suggested that PCR is a more sensitive and specific method for diagnosis of canine ehrlichiosis. Tick infestations, history of tick infestation and hygienic conditions have all been identified as significant risk factors for disease transmission. The hematological parameters of diseased animals, such as TECs, TLCs, and Hb were dramatically reduced. This study concluded that minocycline can remove or decrease circulating *E. canis*, suggesting that it could be a viable alternative to the "gold standard" doxycycline in present local circumstances.

Availability of Data and Materials

The authors declare that the experimental data supporting the present study findings have been made available to the corresponding author (A. H. Rabbani).

Ethical Consideration

Approval of this study was obtained from the University of Veterinary and Animal Sciences, Lahore, Thesis Committee (Approval no: 8226).

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Competing Interests

There was no conflict of interest with respect to authors reporting their research findings.

Author Contributions

Experimental design was conceived by SA, FAA, AZ and SNA. Data were collected by SA, YRK, ON and MS. Statistical analysis was conducted by AA and KH. Original draft was written by SA, and AHR. All authors have contributed to the revision and final proof-reading of the manuscript.

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