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Comparative diagnostic methods for canine ehrlichiosis

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Abstract: The efficacy of blood and buffy coat smear examination, commercially available dot-enzyme-linked immunosorbent assay (ELISA) kit, and nested polymerase chain reaction (PCR) was evaluated in the diagnosis of canine ehrlichiosis in 40 dogs exhibiting symptoms suggestive of the disease. The intracytoplasmic rickettsial organisms were seen in the smears of only 1 dog (2.5%) with an extremely low level of parasitemia. Nested PCR with 16S rRNA revealed characteristic 378 bp bands indicating positive reaction in 8 (20.0%) dogs and sera samples of 21 (52.5%) dogs revealed anti-*Ehrlichia* antibodies. The dog that revealed organisms in the blood also showed positive results with the other 2 methods. Similarly all the sera samples of dogs with nPCR positive results were also positive for anti-*Ehrlichia* antibodies. The 8 dogs that exhibited positive results by nPCR and dot-ELISA were studied for clinical, hematological, and biochemical characterization of the disease. Fever, depression, anorexia, anemia, splenomegaly, and bleeding tendency were the common symptoms associated with the disease. Thrombocytopenia was the most consistent hematological finding, followed by anemia. Biochemical analysis revealed hyperglobulinemia with a reverse albumin:globulin ratio in 3 cases.

Key words: Dog, *Ehrlichia canis*, blood smear, hematology, biochemical analysis, clinical signs, ELISA, PCR

1. Introduction

Ehrlichia canis, an etiological agent of canine monocytic ehrlichiosis or canine hemorrhagic fever, is a small gram-negative coccoid bacterium that resides and replicates in the cytoplasm of circulating monocytes and macrophages. *E. canis* infection in dogs and other canine hosts is transmitted biologically by a common dog tick, *Rhipicephalus sanguineus*, in which only transstadial transmission is reported (1). Although the disease has a global distribution, it is widely prevalent in tropical and subtropical countries, including India. Among the 4 species of *Ehrlichia* parasitizing dogs, *E. canis* has been reported frequently, while thrombocytic *E. platys* (2) and granulocytic *Anaplasma phagocytophilum* (3) have been reported sporadically from different parts of India.

The incubation period of canine ehrlichiosis ranges from 8 to 20 days, after which the clinical entity reveals 3 phases: acute, subclinical, and chronic (4). The commonly encountered signs such as fever, splenomegaly, lymphadenopathy, anemia, hemorrhages on mucus membranes, and conjunctiva and bleeding through natural orifices in the form of epistaxis, hematemesis, hematuria, and melena vary considerably in severity and frequency of occurrence in the initial and terminal phases of infection, which are well separated by a prolonged subclinical phase. Since the prognosis of the last 2 phases is invariably guarded to grave, early confirmative diagnosis of the disease is urgently required to prevent life threatening or fatal outcome (5).

Currently diagnosis of canine ehrlichiosis is based on anamnesis, clinical presentation, and confirmatory laboratory investigations, which include 2 conventional, (demonstration of organisms in blood smears and in vitro cultures), immunological (demonstration of circulating antibodies), and molecular (demonstration of genus and species specific DNA by polymerase chain reaction) approaches. Microscopic demonstration of intracytoplasmic organisms in monocytes is diagnostic for the disease, but very few blood samples reveal the organisms owing to low levels of parasitemia. The cell culture reisolation method is reported to be very sensitive and definitive, but is not a convenient method as it requires 14–34 days to give results and thus defeats the whole purpose of early diagnosis (6). Serological methods including indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and western blot (WB) assay, are effective for detection of antibodies to *E. canis*, but failure of the techniques to differentiate current and past infections and early infections (1–3 weeks postinfection), limit their reliability as a confirmative indicator of the disease.

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Polymerase chain reaction (PCR) based detection of *E. canis* though gaining acceptability within the scientific community to overcome the problems listed above requires sophisticated laboratory and accurate standardization. Owing to this, the results are subjectively interpreted as there may be inter- and intralaboratory variations that, at present, limit the applicability of the tool in the diagnosis of the disease. Nevertheless, the molecular approach has shown encouraging promise not only in the diagnosis but also in epidemiological studies and analysis of anti-*Ehrlichial* chemotherapy (7). Thus diagnosis of the disease particularly in the subclinical phase poses a serious challenge for practicing veterinarians. The present work was therefore undertaken to evaluate laboratory procedures available for diagnosis of canine ehrlichiosis. Since immunological and molecular methods are not available uniformly throughout the country, efforts were also made to find out hematological and biochemical alterations that help in the clinical assessment of canine ehrlichiosis.

2. Materials and methods

The dogs presented to pet practitioners in and around Mumbai and Bai Sakarbai Dinshaw Petit Hospital, Mumbai, showing at least 2 characteristic clinical signs of canine ehrlichiosis, which include high fever, splenomegaly, bleeding from natural orifices, hemorrhages on mucus membranes, and presence of ticks on the body, were included in the study. The description of animals and the clinical history of these dogs were noted. Blood/sera samples of suspected dogs were subjected to parasitological (01/40), immunological (21/40), molecular (08/21), hematological (08/08), and biochemical investigations (08/08).

2.1. Blood/buffy coat smear examination

Peripheral blood smears and buffy coat smears were stained with Field stain and Giemsa stain as per the technique described by Rathore and Sengar (8) for the demonstration of intracytoplasmic rickettsiae.

2.2. Immunological analysis

For analysis 10 µL of blood or 5-µL sera samples from suspected dogs were subjected to dot-ELISA using a commercially available kit (ImmunoComb, Biogal, Galed Laboratories, Israel) and following the manufacturer's protocol.

2.3. Molecular diagnosis of *E. canis*

In order to improve the efficacy of the test and prevent interference of hemoglobin in PCR (9), 200 µL of buffy coat of each sample was used for extraction of 16S rDNA. For obtaining this 200 µL of buffy coat, 2 mL of blood was centrifuged. The DNA was extracted by using a Genei pure blood genomic DNA purification kit (Bangalore Genei, India), according to the manufacturer's protocol, but the sample was incubated overnight in lysis buffer I.

2.3.1. Standard polymerase chain reaction

The PCR protocol as described by Lakshmanan (10) was followed with a few modifications. A 477 base pair fragment of 16S rRNA gene was amplified using previously published genus specific **FECC:** 5′ AGA AGG AAC GCT GGC GGC AAG C 3′ and **RECB:** 5′ CGT ATT ACC GCG GCT GCT GGC A 3′, primers (8). Amplification of genus specific reaction was carried out in a 25-µL PCR tube containing 11.0 µL of dH_2O, 2.5 µL of 10X buffer without $MgCl_2$, 1.2 µL of $MgCl_2$ (mM), primers (30 pmol) 1.0 µL each, 3.0 µL of dNTPs (2.5 mM), and 0.3 µL of Taq polymerase. Then 5 µL of DNA template was added to the master mix. Initial denaturation was done at 94 °C for 4 min followed by annealing at 62 °C for 1 min and extension at 72 °C for 2 min. After that, 35 cycles at 94 °C for 1 min, 62 °C for 45 s and 72 °C for 1 min were done. A final extension at 72 °C for 10 min was done.

2.3.2. Single tube nested polymerase chain reaction

The primers used for species specific nested PCR were **FP:** 5′-GTG GCA GAC GGG TGA GTA ATG C 3′ and **RP:** 5′-CAG AGT TTG CCG GGA CTT CTT C 3′. The amplification reaction was carried out in a 25-µL PCR tube with the following protocol: 15.0 µL of dH_2O, 2.5 µL of 10X buffer without $MgCl_2$, 1.2 µL of $MgCl_2$ (mM), 1.0 µL of species specific primers (30 pmol each), 3.0 µL of dNTPs (2.5 mM), and 0.3 µL of Taq polymerase to which 1 µL of amplicons of genus specific reaction was added. The cycling conditions followed were the same as for the genus specific reaction. Species specific PCR amplicons obtained at 378 bp were cut from agarose gels and purified using GeneiPureTM Quick PCR Purification Kit (Bangalore Genei, India) according to the manufacturer's recommendations. DNA sequencing was performed in both directions by Bangalore Genei. Sequence chromatograms were read and analyzed.

2.4. Hematology and biochemical analysis of blood and sera samples

Whole blood samples of confirmed cases were subjected to blood urea nitrogen (BUN) estimation as per the method described by Benjamin (11). Sera samples of confirmed *Ehrlichia* cases were subjected to a liver function test (Alanine aminotransferase, aspartate aminotransferase, and serum bilirubin) and kidney function test (blood urea nitrogen and serum creatinine) as per the method described by Benjamin (11).

2.5. Clinical characterization of canine ehrlichiosis

The dogs that tested positive for canine ehrlichiosis either by blood smear/buffy coat smear examination or by commercially available ELISA kit and PCR technique were monitored closely. The dogs were subjected to thorough physical examination and case history and clinical signs exhibited by the dogs were recorded systematically.

3. Results

In the present study, 40 clinical cases of suspected canine ehrlichiosis exhibiting at least 2 of the classical symptoms were included for diagnostic, clinical, hematological, and biochemical characterization of canine monocytic ehrlichiosis in the Mumbai region

3.1. Blood and buffy coat smear examination

In the current investigation, peripheral blood and buffy coat smears of 40 cases suspected for canine ehrlichiosis were prepared in duplicate and stained with the Giemsa and Field methods. Only 1 dog (2.5%) showed intracytoplasmic organisms in the mononuclear cells. The organisms were stained purple and measured 0.5 to 1.5 µm (Figure).

3.2. Immunological analysis

Serum of 21 samples (52.5%) revealed positive results as demonstrated by development of a darker intensity of the color of the test spot as compared to the reference spot on the comb. Eleven dogs showed a high positive reaction, 9 revealed medium titer, and 1 dog exhibited a low positive reaction. The dog that revealed organisms in the blood smears had a high positive ELISA titer. The 20 dogs that did not reveal organisms in the blood/buffy coat smears showed positive indication of the disease by ELISA. Thus the agreement between the 2 methods in the diagnosis of canine ehrlichiosis was found to be 4.76%.

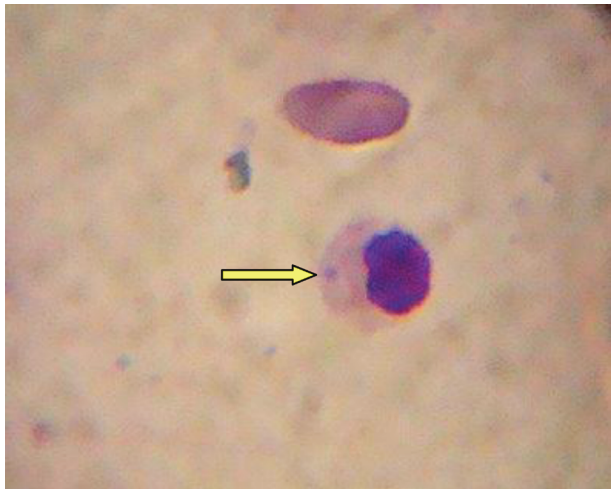

Figure. Intracytoplasmic rickettsiae in lymphocyte.

At present, the diagnosis of canine ehrlichiosis is based on dot-ELISA, since it is the only tool available for widespread application. Hence, the results obtained in the current investigation were also analyzed to note breed, age, and sex predisposition. However, in the present study no such trend was noted (Table 1). Analysis of results of dot-ELISA with respect to age of the dogs (Table 2) revealed a higher occurrence in adult dogs of 1 to 8 years old,

Table 1. Breed-wise predisposition to canine ehrlichiosis.

Breed	No. of dogs examined	No. of positive dogs
	Small breeds	
Lhasa Apso	1	1 (100%)
Pug	3	1 (33.33%)
Pekingese	1	1 (100%)
Maltese	1	0 (00.0%)
Spitz	6	5 (83.33%)
Cocker Spaniel	5	2 (40.0%)
French Bull dog	1	0 (00.0%)
Total	18	10 (55.55%)
	Large breeds	
Labrador	6	4 (66.66%)
Mastiff	2	2 (100%)
German Shepherd	8	2 (25.0%)
Golden Retriever	2	1 (50.0%)
Total	18	9 (50%)
Nondescript	4	2 (50%)
Grand total	40	21 (52.5%)

Table 2. Age-wise predisposition to canine ehrlichiosis.

Age	No. of dogs examined	No. of positive dogs
Young (below 1 year)	11	4 (36.36%)
Adult (1 to 8 years)	23	14 (60.86%)
Old (above 8 years)	6	3 (50%)
Total	40	21 (52.5%)

followed by senile dogs, and it was distinctly low in young dogs below 1 year old. Moreover, the disease was found to be more common in male as compared to female dogs (Table 3), with no specific breed predisposition (Table 1).

3.3. Molecular diagnosis of canine ehrlichiosis by PCR

Only 3 samples (7.5%) showed positive reaction on amplification with genus specific primers. Owing to the poor sensitivity of standard PCR, conventional and single step nested PCR was attempted on the genus specific amplicons using the species specific primers. The band of species specific amplification obtained at 387 bp was subjected to DNA sequencing and was homologous to GenBank accession number JN121380.1. The nPCR gave better results as compared to conventional PCR (Table 3). In the present work, 8 (20%) of the 40 samples revealed a positive reaction. The dog that revealed presence of the organisms in the

Table 3. Results of diagnostic methods employed for canine ehrlichiosis (n = 40).

Case no.	Blood/buffy coat smear examination	ELISA titer	nPCR diagnosis
1	–	1:320–1:1280	√
2	–	1:80–1:160	√
3	–	1:160–1:320	√
4	–	1:80–1:160	√
5	–	1:320–1:1280	√
6	–	1:80–1:160	–
7	–	1:320–1:1280	–
8	–	1:320–1:1280	–
9	–	1:80–1:160	–
10	–	1:320–1:1280	–
11	–	1:320–1:1280	–
12	–	1:320–1:1280	–
13	–	1:160–1:320	–
14	–	1:80–1:160	–
15	–	1:20–1:40	–
16	–	1:320–1:1280	√
17	–	1:320–1:1280	–
18	√	1:320–1:1280	√
19	–	1:160–1:320	–
20	–	1:80–1:160	√
21	–	1:320–1:1280	–
Total	01 (2.5%)	21 (52.5%)	8 (20.0%)

blood samples was also positive by nested PCR. Similarly all 8 dogs that showed positive PCR results were also positive for anti-*Ehrlichia* antibodies as revealed by dot-ELISA. The quantitative analysis of dot-ELISA titers of dogs with nPCR positive results revealed that 4 of the 8 samples had high antibody titers, 3 had medium positive titers, and 1 dog had a low antibody titer. Thus, there was 38.09% agreement between nPCR and ELISA results and 12.5% agreement between nPCR and blood/buffy coat smear examination.

3.4. Clinical characterization of canine ehrlichiosis

The 8 cases that revealed positive results with dot-ELISA and nPCR were selected for clinical characterization of canine monocytic ehrlichiosis. The parameters included for the purpose were categorized in 2 groups: clinical signs and physical examination.

Depression and anorexia were the primary concerns of most of the pet owners at the time of presentation of the case to the veterinarians. Bleeding tendencies in the form of epistaxis, hematuria, and melena were also encountered in 50% of the dogs. Physical examination revealed fever in 100% cases, splenomegaly in 62.5% cases, paleness of visible mucus membranes indicating anemia in 52.5% cases, and petechial/ecchymotic hemorrhages on oral mucosa, penis, and conjunctiva in 50% cases of confirmed canine ehrlichiosis. Ticks were encountered on the body in 50% of the dogs.

3.5 Hematological and biochemical profiles in canine monocytic ehrlichiosis

3.5.1 Hematological profile

The most prominent feature noted in the study was thrombocytopenia as all the dogs (100%) revealed values below the normal range. The values of total erythrocyte count, hemoglobin percentage, and packed cell volume of 4 (50%) out of the 8 dogs specified in Table 4 were on the lower side of the reference scale, indicating an anemic trend, which corresponds precisely with the clinical findings noted in this study. Three dogs (case no. 2–4) had total erythrocyte counts below the normal range; 4 dogs (case no. 1–4) showed subnormal hemoglobin percentages, and 1 dog had a low packed cell volume. The analysis of anemia based on erythrocytic indices such as mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration revealed that 1 dog (case no. 4) had a normochromic normocytic (nonregenerative) trend and the remaining 3 dogs (case no. 1–3) showed hypochromic anemia; 1 dog (case no. 1) also revealed a microcytic trend. The remaining 4 dogs (case no. 5–8) did not reveal anemia. Leukocytosis was evident in 5 (62.5%) out of the 8 dogs. Differential leukocytic counts of these 5 cases exhibited relative lymphocytosis in 2 cases, and relative neutrophilia in 1 case (Table 5).

Table 4. Hematological profile (erythrocytic) of canine ehrlichiosis.

Case no.	Temp. (F)	TEC (10^6/µL)	Hb (g/dL)	PCV (%)	MCV (fL)	MCHC (g/dL)	Remarks
1	219.2	6.30	9.5	43.18	50.9	22.00	Hypochromic microcytic anemia
2	224.6	4.17	9.0	28.4	68.1	21.60	Hypochromic normocytic anemia
3	217.4	3.14	7.1	24.5	72.0	28.90	Hypochromic normocytic anemia
4	217.4	4.52	10.2	32.2	71.23	31.68	Normochromic normocytic anemia
5	224.9	8.30	16.4	51.5	64.0	31.9	–
6	224.6	5.37	12.4	38.7	72.6	32.4	–
7	219.9	7.71	17.9	51.2	66.40	34.96	–
8	217.4	7.9	15.4	38.7	54.9	32.1	–
Range (Avg.)	217.4 – 224.9	3.14–8.30 (5.92)	7.1–17.9 (12.23)	24.5–51.5 (38.54)	50.9–72.0 (65.01)	21.6–34.96 (29.44)	-
Reference values	101–102	5.5–8.5	12–18	37–55	60–77	31–34	

Table 5. Hematological profile (thrombocytic and leukocytic) of canine ehrlichiosis (n = 8).

Case no.	TLC ($\times10^3$/μL)	Differential leukocyte count (%)					Thrombocyte ($\times10^5$/μL)	Remarks
		L	N	M	E	B		
1	6.9	45	49	01	05	0	0.11	Thrombocytopenia
2	27.4	26	73	01	0	0	0.38	Thrombocytopenia, leukocytosis
3	38.3	07	82	10	01	0	0.11	Thrombocytopenia, leukocytosis, neutrophilia
4	18.7	23	69	01	07	0	1.54	Thrombocytopenia, leukocytosis
5	9.07	18	64	10	08	0	0.68	Thrombocytopenia
6	22.2	32	62	03	03	0	0.80	Thrombocytopenia, leukocytosis
7	12.6	13	85	01	01	0	0.02	Thrombocytopenia
8	20.5	32	62	01	05	0	0.28	Thrombocytopenia, leukocytosis
Range (Avg.)	6.9–38.3 (19.46)	7-45 (24.5)	49–85 (68.25)	1–10 (3.5)	0–8 (3.75)	0	0.02–1.54 (0.49)	-
Reference values (Avg.)	6–18	12–30	60–77	3–10	2–10		2–5	-

3.5.2. Biochemical profile

The same lot of blood and sera samples from confirmed *Ehrlichia* cases was subjected to estimation of enzymes and other components that denote liver and kidney functions. Bilirubin, SGPT, and SGOT levels of all the dogs were well within the normal range barring 1 or 2 exceptions with marginal increase. Alkaline phosphatase was found to be elevated in 4 cases. Although hepatomegaly has been reported to be associated with this infection, it is not a constant finding that characterizes the disease. Owing to normal levels of serum bilirubin, it can be concluded that the anemia noted in the present study was not a primary result of destruction of erythrocytes. As regards protein estimation, it was evident that 5 out of the 8 dogs exhibited normal values. Kidney profiles, represented by levels of blood urea nitrogen (BUN) and serum creatinine, of the 8 dogs included in the study did not reveal any conclusive trend though 1 dog showed slight increase in BUN.

4. Discussion

Canine ehrlichiosis, a common entity in dogs in India including Mumbai, is prima facie diagnosed on the basis of clinical signs of fever, splenomegaly, hemorrhages on visible mucosa, and bleeding tendency. However, these clinical signs are also seen in other infectious diseases like leptospirosis and other conditions; therefore differential diagnosis is essential for proper curative and prophylactic treatment. At present, in most of the laboratories in India, the emphasis is given to either demonstration of the organisms in blood or buffy coat smears or use of commercially available dot-ELISA kit for detection of anti-*Ehrlichia* antibodies. However, both these techniques have their own limitations that pose a serious problem for clinicians to recognize the exact status of the infection and prognosis. In the present study, the morphometric features of the organisms matched the description of *E. canis* (12–14). However, the level of parasitemia was extremely low. Similar observations of low detection rate and low levels of parasitemia were also recorded by Woody and Hoskins (15), Juyal et al. (16), Thriunavukkarasu et al. (17), Waner et al. (5), and Nakaghi et al. (18), in spite of the exhibition of typical signs of the disease. Previous authors reported as low as 0.2% parasitemia in dogs infected with *E. canis*, which means 1 out of 500 circulating leukocytes was infected with the organisms. On the other hand, Katyal (19), Lakshmanan (10), Mallapur (20), and Samaradhni et al. (21) reported a higher detection rate as revealed by 17.58%, 55%, 18.9%, and 5.86% prevalence, respectively.

This discrepancy in the detection of rickettsia might be due to the clinical phase of the infection, tenacity of laboratory personnel to inspect large numbers of cells in each smear, and occurrence of other diseases with similar symptomatic expression. Thus, demonstration of organisms in blood samples, though confirmative, is not a reliable method of diagnosis (9,15,18).

It was also noted that the prevalence (19) as well as severity of clinical signs (19,22) was higher in German Shepherds. Thriunavukkarasu et al. (23) and Lakshmanan (10) noted a higher prevalence in pure breed dogs as compared to nondescript local breeds. This discrepancy in the breed predisposition might be due to a number of factors such as nutritional status, immunological competence, and susceptibility of different breeds to tick infestation (24). In our study, we did not note any such trend. However, we found the incidence of canine ehrlichiosis to be higher in males as compared to females, whereas Lakshmanan (10) reported a reverse trend.

Similarly, dot-ELISA based on antibody detection does not diagnose early infection and differentiate current and past infections. The trend of disparity between the 2 diagnostic tools, the conventional and immunodiagnostic methods, is obviously due to low levels of parasitemia leading to detection of less number of cases. In contrast, ELISA, being one of the most sensitive assays that detects even very low levels of antibody titers, showed a positive reaction in a higher number of cases. Nevertheless, higher detection rates elicited by dot-ELISA in the present study could also be attributed to 2 vital factors; false positive results due to cross reactivity (25) and failure of the assay to differentiate current and past infection (26,27). One more drawback reported to be associated with dot-ELISA or any other immunodiagnostic methods is failure of the tool to detect early infection. McBride et al. (28) remarked that IgM and IgG antibodies are not detectable until at least 1 to 3 weeks postinfection.

The 16S rRNA gene fragment that was amplified in this study was also exploited by Iqbal et al. (6), McBride et al. (28), Inokuma et al. (29), and Unver et al. (30) with reasonably reliable results. The negative nPCR sample (n = 15) may also be explained by the capacity of the organisms to "hide" in splenic macrophages (4). Although all the cases included in the present study were clinical, there was every possibility that some of these may represent past infection as it takes a considerably long time to alleviate some of the symptoms such as splenomegaly and anemia. Nevertheless, some of these symptoms may be due to other etiological origins (leptospirosis, hemoprotozoa, etc.) as revealed by the fact that 19 (47.5%) out of the 40 cases included in the study showed negative results by all the 3 diagnostic procedures. This scenario also indicates that clinical diagnosis has its own limitations and thus warrants the use of PCR for recognition of the active phase of the infection (5,6). Previous authors opined that, owing to convenience and direct indication of active infection, PCR is more suitable for reliable diagnosis. In contrast, dot-ELISA, though user friendly and quick, may not detect early infection due to inadequate immunostimulation, leading to false negative results, and may not rule out past infection due to persistence of antibodies in the circulation, leading to false positive results. In the present work, none of the 19 dogs with dot-ELISA negative results showed nPCR positive results and thus superiority of PCR over serodiagnostic approach though anticipated was not proved. Overall comparison of the results of the 3 diagnostic methods evaluated in this work clearly indicates that PCR-based diagnosis has the ability to overcome the problem of underdetection of cases by blood smear/buffy coat examination as well as the problem of overdetection of the cases by dot-ELISA. In addition, the technique also has very good prognostic value and can be employed for reliable judgment of chemotherapeutic efficacy. In the future, sensitivity, specificity, and simplicity of PCR can be further improved by implementing modifications like real-time PCR and more significantly multiplex PCR for combined differential diagnosis of blood and rickettsial infections in dogs.

4.2. Hematological and biochemical profile

All the dogs included in the study showed thrombocytopenia. However, thrombocytopenia, though an important hematological finding, does not rule out other diseases like leptospirosis and other conditions. Three reasons have been put forth in the literature to explain development of thrombocytopenia. Smith et al. (31) suggested that thrombocytopenia in *E. canis* infection is mainly due to large-scale destruction of the cells in the spleen that begins a few days after the infection, but Waner (32) pointed out that bone marrow hypoplasia leading to impairment of normal functions is the primary cause of pancytopenia including thrombocytopenia. The development of thrombocytopenia has also been attributed to an immunopathological mechanism by Waner et al. (5), who demonstrated significant levels of serum antiplatelet IgG, 17 days after experimental *E. canis* infection that resulted in the removal of antibody adsorbed thrombocytes by the mononuclear phagocyte system in the liver and spleen. The authors hypothesized that *E. canis* infection in dogs altered the immune system, resulting in overproduction of natural antiplatelet antibodies of increased affinity. Thus, owing to a severe drop in the cell count, it appears that the presence of antiplatelet antibodies is one of the major causes of thrombocytopenia; involvement of nonimmunological mechanisms may also contribute in aggravating the condition.

The nonregenerative anemia, noted in a solitary case in the present study and also recorded by Thriunavukkarasu et al. (23) and Waner et al. (26), might be due to bone marrow hypoplasia leading to impaired production of cellular components of blood. The variation in the type of anemia can be attributed to several influential factors such as nutritional status, iron reserves in the body, concurrent infection, and age of the infected dogs. Total leukocytic counts of the 8 cases ranged from 6900 to 38,300/µL (average 19,458/µL). Three cases showed hyperglobulinemia with a reverse albumin:globulin ratio probably due to increased levels of gamma globulins in long-standing cases (5).

5. Conclusion

nPCR detected a greater number of cases than blood smear examination and a lower number of cases than dot-ELISA, which appeared to be logical owing to the extremely low level of parasitemia in blood smears and persistence of circulating antibodies of past infection. In addition, the ability of the technique to identify the active phase of infection is useful for reliable prognosis of the disease and judgment of chemotherapeutic efficacy. However, the constraints of time consumption, tedious steps, and availability of thermal cycler limit its routine use.

Clinical analysis of the cases revealed that, though prolonged depression, anorexia, and frequent episodes of pyrexia were the presenting signs, the disease exhibits its presence with a recurrent bleeding tendency and hemorrhages on the visible mucosa. However, laboratory investigation is mandatory for differential diagnosis.

Hematological analysis of *Ehrlichia* confirmed cases revealed thrombocytopenia in all the dogs. The other findings noted inconsistently in the study were anemia of different types and leukocytosis with relative deviation of the differential leukocyte counts.

Biochemical profiles of the dogs with confirmed ehrlichiosis showed hyperglobulinemia with a reverse albumin-globulin ratio in 3 cases. All other parameters indicating liver and kidney functions did not reveal a conclusive trend.

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