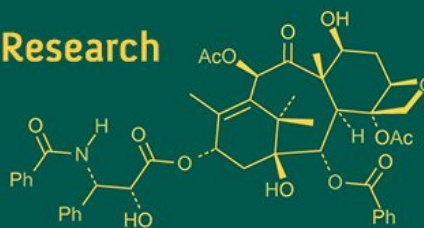


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Efficacy of *Crotalus horridus* as an adjunct therapy in canine babesiosis

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Abstract

Canine babesiosis caused by *Babesia gibsoni* is a clinically significant hemoprotozoan disease characterized by hemolytic anemia, persistent thrombocytopenia, and frequent relapse after treatment. Conventional therapeutic regimens often improve clinical signs but may not ensure sustained parasitological clearance. The present scientific article evaluates the comparative therapeutic efficacy of *Crotalus horridus* 200C as an adjunct therapy to standard antimicrobial treatment in naturally occurring *Babesia gibsoni* infection. A total of 74 clinically suspected dogs were screened, of which 30 PCR-confirmed *B. gibsoni* positive dogs were randomly allocated into three treatment groups: Group A received doxycycline-enrofloxacin-metronidazole, Group B received the same regimen with adjunct *Crotalus horridus* 200C, and Group C received diminazene aceturate. Comparative assessment was based on clinical recovery, hematobiochemical restoration, peripheral smear examination, and PCR negativity on days 0, 7, and 21. Group B demonstrated the best therapeutic response with 100% clinical recovery, faster normalization of hemoglobin, packed cell volume, and platelet counts, 80% PCR clearance, and absence of relapse by day 21. The findings indicate that *Crotalus horridus* acts as an effective adjunct in accelerating hematological and clinical recovery in canine *Babesia gibsoni* infection.

Keywords: *Babesia gibsoni*, canine babesiosis, *Crotalus horridus*, adjunct therapy, thrombocytopenia, comparative efficacy

Introduction

Canine babesiosis is an important tick-borne hemoprotozoan disease caused by intraerythrocytic parasites of the genus *Babesia*, among which *Babesia gibsoni* is especially important due to its chronicity and high relapse rate. The disease may occur in acute, chronic, or subclinical forms and is primarily associated with fever, lethargy, hemolytic anemia, thrombocytopenia, and hepatic dysfunction. Persistent thrombocytopenia is considered one of the most consistent hematological hallmarks of *B. gibsoni* infection (Kettner *et al.*, 2003) [16]. Conventional therapies include diminazene aceturate, imidocarb dipropionate, clindamycin-based combinations, and doxycycline-enrofloxacin-metronidazole protocols. However, no single treatment protocol has proven completely effective in eliminating the parasite, and many dogs remain chronic carriers with recurrent parasitemia (Jefferies *et al.*, 2003) [13]. Because thrombocytopenia and hemorrhagic tendencies are major prognostic concerns, adjunct therapies capable of accelerating platelet recovery may improve clinical outcomes. *Crotalus horridus*, a homeopathic preparation traditionally indicated in hemorrhagic diathesis, jaundice, black vomit, and vascular disorders, has shown encouraging responses in canine ehrlichiosis and dengue-associated thrombocytopenia (Tungnunga *et al.*, 2016; Nayak *et al.*, 2019) [20, 28].

Therefore, the present study was undertaken to comparatively evaluate the efficacy of *Crotalus horridus* as an adjunct therapy in naturally occurring canine babesiosis.

3. Materials and Methods

The study was conducted in the Department of Veterinary Medicine of Krantisinh Nana Patil College of Veterinary Science, Shirwal, Satara from May 2025 to January 2026. A total of 74 suspected dogs were examined for *Babesia gibsoni* infection based on the clinical signs such as fever, pale or icteric mucous membranes, anemia, tick infestation, dark yellow urine,

Swollen lymph node and hemoglobinuria. In order to confirm the diagnosis of *Babesia gibsoni* infection, the infected dogs in this study underwent clinical examination and diagnostic procedures such as Giemsa's stain peripheral blood smear testing, and polymerase chain reaction.

About 5 mL blood sample was collected from each dog using a sterile disposable syringe via either cephalic or lateral saphenous venipuncture. Of this, approximately 3 mL was transferred into a K₂EDTA (ethylenediaminetetraacetic acid) vial for hematological analysis, blood smear examination, and genomic DNA extraction for polymerase chain reaction (PCR) testing to detect *B. gibsoni* DNA. The remaining 2 mL of blood, collected without anticoagulant, was placed in a separate vial for serum separation and biochemical analysis. Hematological parameters includes Hb (g/dl), Total Erythrocyte Count ($\times 10^6/\mu\text{L}$), Packed Cell Volume (%), Mean Corpuscular Volume (fl), Mean Corpuscular Hemoglobin Concentration (g/dL), Total Leucocyte Count ($\times 10^3/\mu\text{L}$), Differential Leucocyte Count (%) and Platelets Count (Lacs/ μL). Abacus Junior vet 5 part machine were used for analysing hematological parameter before treatment on day 0 and after treatment on day 7 and

21. Serum samples were obtained by centrifuging the collected blood at 2500 rpm for 3 minutes. The separated serum was carefully transferred into sterile microtubes and stored at -20°C until further biochemical analyses were performed. Biochemical parameters includes Total Bilirubin (mg/dl), Direct Bilirubin (mg/dl), Indirect Bilirubin (mg/dl), Total Protein (g/dl), Albumin (g/dl), Globulin (g/dl), Alanine Aminotransferase (U/L), Aspartate Transferase (U/L), Blood Urea Nitrogen (mg/dl), Creatinine (mg/dl) and Alkaline Phosphatase (U/L). Biochemical evaluations were conducted using alta chem semi-automated clinical chemistry analyser (ADX-CHEM-220) before treatment on day 0 and after treatment on day 7 and 21.

The molecular confirmation of the *Babesia gibsoni* parasites was carried out by PCR. DNA was extracted from the blood sample by applying the Phenol Chloroform method. Extracted DNA sample is used as template to amplify the fragment of *Babesia gibsoni* for 18S rRNA using the primers described by Inokuma *et al.* (2004) ^[10]. Specification of the primers are given below in the table no. 3.1

Table 3.1: *Babesia gibsoni* primer specification used in PCR

Sr. No.	Name	Primer Sequence	Product Size
1.	Gib 599	CTC GGC TAC TTG CCT TGT C	670 bp
2.	Gib 1270	GCC GAA ACT GAA ATA ACG GC	

The PCR reaction mixture was prepared using a 2× Taq Master Mix containing Taq DNA polymerase, reaction buffer, dNTPs, and additional stabilising agents in optimised concentrations. Composition of reaction mixture of PCR is displayed in table no. 3.2

Table 3.2: Reagents used in making PCR reaction mixture

Sr No.	Reagents	Volume
1.	Master Mix (Takara GT PCR mix)	12.5 μL
2.	Forward Primer	1 μL
3.	Reverse Primer	1 μL
4.	DNA	3 μL
5.	Nuclease Free Water (NFW)	7.5 μL
Total Volume		25 μL

Above mentioned mixture are well prepared and mixed by vortexing and placed in the thermal cycle with specified cycle setting. Cycling condition for amplification is shown in table no. 3.3.

Table 3.3: Cyclic condition for amplification of DNA segment

<i>Babesia gibsoni</i> group	Steps	Temperature	Time	Cycles
	Initial Denaturation	95 $^\circ\text{C}$	5 min	35
	Denaturation	95 $^\circ\text{C}$	30 sec	
	Annealing	55 $^\circ\text{C}$	30 sec	
	Extension	72 $^\circ\text{C}$	90 sec	
	Final Extension	72 $^\circ\text{C}$	5 min	

Extracted DNA was analyzed by agarose gel (1.5%) electrophoresis and visualization of band were done under UV transilluminator.

30 *Babesia gibsoni* positive cases were equally distributed into 3 equal groups (n=10). Group A was treated with Inj. Doxycycline @10 mg/kg IV SID for 21 days, Inj. Metronidazole @15mg/kg IV on first day followed by 8 mg/kg IV SID for next 20 days and Inj. Enrofloxacin @5 mg/kg IM SID for 21 days, Group B was treated with Inj. Doxycycline @10 mg/kg IV SID for 21 days, Inj.

Metronidazole @15mg/kg IV on first day followed by 8 mg/kg IV SID for next 20 days, Inj. Enrofloxacin @5 mg/kg IM SID for 21 days and Liq. *Crotalus horridus* 200C 5 ml Sub-lingual BID for 21 days and Group C was treated by Inj. Diminazene aceturate @3.5 mg/kg deep IM.



Plate 3.1: Blood collection from Cephalic vein in dogs



Plate 3.2: Thermometer showing high rectal temperature in dog

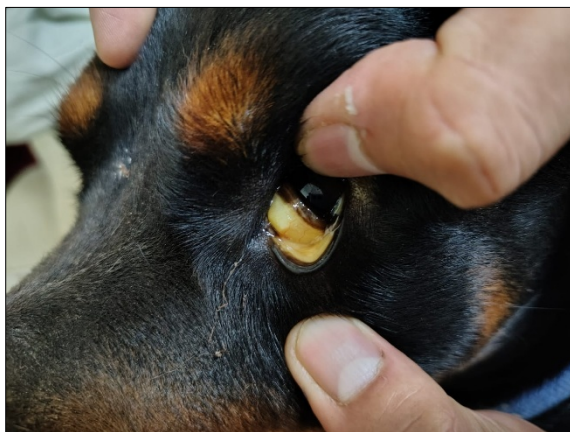


Plate 3.3: Icteric mucous membrane in *Babesia gibsoni* infected dog



Plate 3.4: Pale mucous membrane in *Babesia gibsoni* infected dog

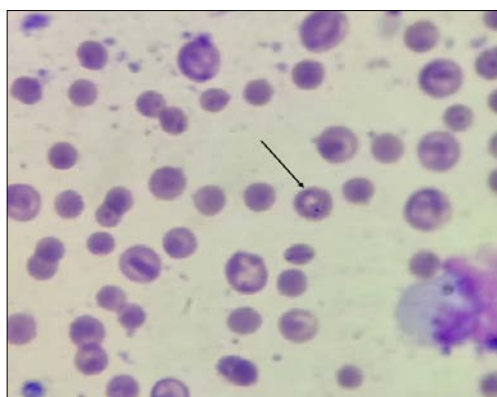


Plate 3.5: Blood smear showing intra-erythrocytic piroplasm of *Babesia gibsoni*

4. Result and Discussion

4.1 Clinico-physiological parameters

At the beginning of the study or before treatment (Day 0), rectal temperature, heart rate and respiratory rate of group A,B and C was showing no significant difference indicating comparable baseline values.

Rectal temperature decreased significantly in Group B and C from day 0 to day 7 and day 21 ($p < 0.05$). By day 21 Group A (101.67 ± 0.19) and Group B (101.53 ± 0.17) had recorded significantly ($p < 0.05$) lower temperature than Group C (102.39 ± 0.07).

Heart rate had also declined over the period of time. Group A (114.4 ± 8.54) showed significantly ($p < 0.05$) lower heart rate as compare to group C (139.2 ± 4.035) while group B (134.8 ± 6.66) remained intermediate. On day 21 Group A (110.8 ± 6.85) had significantly lowered heart rate from group B (131.6 ± 4.824) and group C (134.2 ± 3.076), which did not differ significantly from each other.

Similarly, the respiratory rate was significantly ($p < 0.05$)

lower in group A (21.2 ± 1.08) as compare to group B (26.6 ± 1.74) and group C (27.6 ± 1.02) on day 7. By day 21 group A (22 ± 0.66) showed had significantly ($p < 0.05$) lowered respiratory rate as compared to group C (29.2 ± 1.44) while group B remain (25.6 ± 1.33) intermediate.

The result in the present studies showed increase in the rectal temperature in *Babesia gibsoni* infected dogs indicating pyrexia associated with acute parasitaemia and systemic inflammatory response. Fever is considered one of the earliest and most consistent clinical manifestations of canine babesiosis. Similar observations were reported by Taboada and Merchant (1991) [27], Furlanello *et al.* (2005) [8], Bourdoiseau (2006) [5] and Irwin (2009) [11]. Elevated heart rate and respiratory rate in *Babesia gibsoni* infected dogs may be attributed to fever, hypoxia secondary to anemia and increased metabolic demand. Similar elevations in heart rate have been documented in dogs affected with babesiosis by Adebayo *et al.* (2016) [1] and Chaudhuri *et al.* (2017) [7].

Table 4.1.1: Clinical Parameters of different treatment groups before and after treatment

Clinical Parameter	Groups	Day 0	Day 7	Day 21
Rectal Temperature	A	102.53 ± 0.43	101.84 ± 0.31	101.67 ± 0.19^a
	B	103.24 ± 0.22^x	101.94 ± 0.16^y	101.53 ± 0.17^{ay}
	C	103.43 ± 0.21^x	102.53 ± 0.10^y	102.39 ± 0.07^{by}
Heart Rate	A	131 ± 8.49	114.4 ± 8.54^a	110.8 ± 6.85^a
	B	139 ± 8.58	134.8 ± 6.66^{ab}	131.6 ± 4.824^b
	C	143 ± 4.063	139.2 ± 4.035^b	134.2 ± 3.076^b
Respiratory Rate	A	23.8 ± 1.41	21.2 ± 1.08^a	22 ± 0.66^a
	B	26.2 ± 1.64	26.6 ± 1.74^b	25.6 ± 1.33^{ab}
	C	28.8 ± 1.31	27.6 ± 1.02^b	29.2 ± 1.44^b

Values with different superscript letters (a, b) are significantly different between groups. Values with different superscript letters (x, y) are significantly different within groups ($p < 0.05$).

4.2 Hematological parameter

A progressive improvement in hemoglobin (Hb), Packed Cell Volume (PCV) and Total Erythrocyte Count (TEC) was recorded from day 0 to day 21 in all the groups, with the highest increase recorded in group B followed by group A, whereas group C showed comparatively lowered improvement. Group B showed significant ($p < 0.05$) increase in Hb, PCV between day 0 and day 21 while significant increase in TEC was observed between day 0 and day 7. Group A also showed significant increase in TEC between day 0 and day 21.

The decrease in Hb, PCV and TEC values in the *B. gibsoni* infected dogs also confirms the anemia due to multifactorial etiology such as direct mechanical damage of RBC membrane by the parasites, splenic removal of the parasitised RBCs and immune mediated destruction of RBC were in close confirmation with the findings Salem and Farag (2014) [22], Jain *et al.* (2017) [12], Yogeshpriya *et al.* (2018) [29], Mittal, *et al.* (2019) [19], Anju *et al.* (2021) [2], Liu *et al.* (2022) [18], Reddy *et al.* (2024) [21], Kaliuzhnyi *et al.* (2025) [14].

Increase in the Hb, PCV and TEC values in *Babesia gibsoni* infected dogs after the treatment due reduced erythrocyte destruction, Stabilization of circulating RBC's, reduction in parasitaemia similar observation were observed by Birkenheuer (2004) [4], Selvaraj *et al.* (2010) [23], Lin and Huang (2010) [17], Lin *et al.* (2012), Shrivastva *et al.* (2014) [25], Karasová *et al.* (2022) [15] and Bhasme *et al.* (2024) [3].

The Mean Corpuscular Volume (MCV) showed slight declining trend in group A, while group B and C exhibited gradual increase by day 21. The Mean Corpuscular Hemoglobin Concentration (MCHC) remained stable throughout the study period with minor fluctuation among the groups.

These MCV and MCHC values showed minor variations but remained within a similar range, with no marked increase or decrease. The minimal alteration in the erythrocyte indices with normocytic normochromic anemia, which is typical of acute hemolytic conditions like *Babesia gibsoni* infection was observed in this study were in close conformation with

the findings of Salem and Farag (2014) [22], Anju *et al.* (2021) [2].

A decreasing trend is Total Leucocyte Count (TLC) was observed in group A, whereas group B and group C showed mild fluctuation with no marked alteration.

After treatment the normalization of TLC count suggested reduction in inflammatory response is in close confirmation with the findings of Lin and Huang (2010) [17], Solano-Gallego and Baneth (2011) [26], Baneth *et al.* (2015) [31] and Karasová *et al.* (2022) [15].

Platelets counts increased significantly ($p < 0.05$) in group B between day 0 to day 7 and day 21. At day 21 platelets counts of group C had showed significant lowered count as compared to group A and group B.

Marked thrombocytopenia were observed in this study in case of *B. gibsoni* infected dogs may result from immune-mediated destruction, splenic sequestration due to splenomegaly or increased consumption similar findings were observed by Furlanello *et al.* (2005) [8], Salem and Farag (2014) [22], Jain *et al.* (2017) [12], Yogeshpriya *et al.* (2018) [29], Anju *et al.* (2021) [2], Liu *et al.* (2022) [18], Reddy *et al.* (2024) [21], Kaliuzhnyi *et al.* (2025) [14].

Increase in the thrombocyte counts suggests improvement in bone marrow function, decrease spleen sequestration and reduction in immune mediated platelets destruction similar observation was reported Chaudhuri and Varshney (2007) [6], Selvaraj *et al.* (2010) [23], Nayak *et al.* (2019) [20] and Game *et al.* (2024) [9].

The significantly faster platelet recovery observed in Group B may be attributed to a combined effect of reduced parasitaemia, decreased immune-mediated platelet destruction, and early stabilization of vascular integrity. In *Babesia gibsoni* infection, thrombocytopenia is multifactorial and commonly results from splenic sequestration, peripheral consumption, endothelial injury, and immune-mediated destruction of circulating platelets. The adjunct use of *Crotalus horridus* 200C possibly accelerated reversal of these mechanisms, thereby promoting earlier restoration of platelet counts compared with conventional antimicrobial therapy alone.

Table 4.2.1: Hematological parameters of different treatment group before and after treatment

Parameters	Groups	Day 0	Day 7	Day 21
Hb (g/dl)	A	7.09 ± 1.06	9.51 ± 1.63	11.19 ± 1.07
	B	7.30 ± 1.08 ^x	10.38 ± 1.04 ^{xy}	12.50 ± 0.86 ^y
	C	8.50 ± 1.26	9.27 ± 1.22	11.07 ± 1.01
PCV (%)	A	19.85 ± 3.10	25.80 ± 4.09	31.09 ± 3.06
	B	19.74 ± 2.73 ^x	27.85 ± 3.20 ^{xy}	33.23 ± 2.08 ^y
	C	24.28 ± 3.82	24.78 ± 3.28	30.45 ± 2.95
TEC (x 10 ⁶ / μl)	A	3.31 ± 0.50 ^x	4.38 ± 0.63 ^{xy}	5.21 ± 0.38 ^y
	B	3.48 ± 0.69 ^x	5.14 ± 0.50 ^y	5.59 ± 0.31 ^y
	C	4.15 ± 0.61	4.21 ± 0.54	5.05 ± 0.44
MCV (fl)	A	61.19 ± 2.80	59.58 ± 3.67	57.08 ± 1.96
	B	56.55 ± 1.66	58.07 ± 2.37	59.03 ± 1.02
	C	49.33 ± 6.67	58.95 ± 1.81	60.04 ± 1.56
MCHC (g/dl)	A	35.07 ± 1.68	36.35 ± 0.93	36.02 ± 0.82
	B	36.58 ± 1.17	38.0 ± 1.05	37.05 ± 1.33
	C	35.06 ± 1.20	37.13 ± 1.05	36.41 ± 0.97
TLC (x 10 ³ / μl)	A	22.82 ± 5.15	20.10 ± 2.97	14.50 ± 2.16
	B	18.65 ± 3.47	13.52 ± 2.09	16.30 ± 2.21
	C	13.85 ± 1.77	13.68 ± 2.97	16.32 ± 3.52
Platelets (Lacs/ μl)	A	2.04 ± 0.33	1.96 ± 0.26	2.64 ± 0.44 ^{ab}
	B	1.33 ± 0.27 ^x	2.49 ± 0.31 ^y	3.37 ± 0.29 ^{ay}
	C	1.83 ± 0.38	1.47 ± 0.36	1.53 ± 0.32 ^b

Values with different superscript letters (a, b) are significantly different between groups. Values with different superscript letters (x, y) are significantly different within groups ($p < 0.05$).

Table 4.2.2: Differential Leucocyte Count of different treatment group before and after treatment.

DLC (%)	Groups	Day 0	Day 7	Day 21
Neutrophils	A	78.80 ± 2.48	79.0 ± 4.28	77.40 ± 2.47
	B	70.0 ± 3.40	75.60 ± 3.63	80.0 ± 1.89
	C	74.10 ± 2.71	79.80 ± 2.11	75.2 ± 4.60
Lymphocyte	A	19.0 ± 2.44	17.80 ± 3.76	20.5 ± 2.34
	B	27.0 ± 2.82	22.50 ± 3.79	19.10 ± 1.82
	C	24.0 ± 3.01	17.10 ± 2.56	22.20 ± 4.32
Monocyte	A	0.50 ± 0.26	0.70 ± 0.33	0.50 ± 0.30
	B	0.40 ± 0.30	0.20 ± 0.13	0 ± 0
	C	0.40 ± 0.30	0.70 ± 0.49	0.80 ± 0.46
Eosinophil	A	1.70 ± 0.68	2.30 ± 0.89	1.70 ± 0.51
	B	2.60 ± 0.83	1.80 ± 0.35	0.90 ± 0.34
	C	1.50 ± 0.52	1.10 ± 0.37	1.80 ± 0.92
Basophil	A	0	0	0
	B	0	0	0
	C	0	0	0

Values with different superscript letters (a, b) are significantly different between groups. Values with different superscript letters (x, y) are significantly different within groups ($p < 0.05$).

The Differential Leucocyte Count (DLC) recorded in different group at day 0, day 7 and day 21 is presented in the table no. Neutrophil percentage remained comparatively stable in group A throughout the study period, whereas Group B showed a gradual increase between day 0 to day 21 (70.0 ± 3.40 to 80.0 ± 1.89). Group C showed mild fluctuation during the experimental period.

Lymphocyte percentage showed minor variation among all the groups. In group B, the values decreased from 27.0 ± 2.82 to 19.10 ± 1.82 at day 21, whereas Group A and C showed only slight fluctuations. Monocyte percentage remained low in all groups, with group B showing a decline to 0% by day 21. Eosinophil values exhibited mild variation, with a decreasing trend in group B and slight fluctuation in Group A and C. Basophils were absent in all the groups throughout the study period.

Many researchers have also reported neutropenia and relative lymphocytosis due to chronic infections such as Salem and Farag (2014) [22], Yogeshpriya *et al.* (2018) [29], Anju *et al.* (2021) [2].

4.3 Biochemical parameters

The serum biochemical parameters of different group at day 0, 7 and 21 are presented in the table no. Total protein, albumin and globulin values showed only minor fluctuation during the study periods in all groups. A comparatively increasing trend in total protein and albumin was observed in group B by day 21.

Total bilirubin, direct bilirubin and indirect bilirubin showed decreasing trend in group B from day 0 to day 21. Significant decrease ($p < 0.05$) was observed in the direct bilirubin values in group B from day 0 to day 7 and day 21. Significant lowering ($p < 0.05$) of total bilirubin and direct bilirubin was observed in group C as compared to group A on day 7 while group B remain intermediate.

In the present study, increase in total and direct (conjugated) bilirubin was observed in *Babesia gibsoni*-infected dogs, consistent with hemolysis and hepatic involvement. However, indirect (unconjugated) bilirubin did not show a significant change. This pattern elevated direct but stable indirect bilirubin has been documented in the study of Nagarjuna *et al.* (2024) [33]. Such findings suggest that although hemolysis produces large amounts of unconjugated

bilirubin, the liver's conjugation mechanisms may still function sufficiently in the early stages of infection, with impaired excretion leading to accumulation mainly of the conjugated fraction.

After treatment decrease in total and direct bilirubin values suggests reduction in hepatic inflammation and normalization of hepatic function similar observation were also reported by Baneth *et al.* (2015) [31], Gonde *et al.* (2015) and Lakshmi *et al.* (2024) [32].

Hepatic enzymes activities revealed mild variation during the study period. ALT and AST values tended to decrease in group B by day 21, suggesting improvement in hepatic status. In this study the increase in the ALT and AST values in dogs infected with *Babesia gibsoni* infection suggest hepatocellular damage secondary to hypoxia and systemic inflammation similar findings were observed by Furlanello *et al.* (2005) [8], Zygnier *et al.* (2007) [30], Mittal, *et al.* (2019) [19], Liu *et al.* (2022) [18], Reddy *et al.* (2024) [21], Kaliuzhnyi *et al.* (2025) [14].

Similarly, ALP values showed marked reduction in group B on day 7 followed by partial recovery by day 21 which remained significantly different ($p < 0.05$) from day 0. Also, ALP value in group B was significant ($p < 0.005$) lowered as compared to group A on day 7.

Serum ALP values remained within normal limits with only mild fluctuations among groups. No significant elevation was observed, suggesting minimal hepatobiliary involvement. Similar unchanged ALP activity in canine babesiosis has been reported by Shah *et al.* (2011) [24] and Zygnier *et al.* (2007) [30]. Thus, ALP may not serve as a sensitive marker of hepatic damage in uncomplicated *B. gibsoni* infection.

Renal function indicators namely BUN and Creatinine, remained within the normal physiological limits with minor fluctuation throughout the study period. Also, the creatinine value of group B was significant ($p < 0.05$) lower as compared to group A on day 7, whereas group C remained intermediate. In this study the mean creatinine and BUN values in dogs infected with *Babesia gibsoni* infection were mildly increased due to dehydration or remained in the normal ranges. This observation is in close confirmation with the findings of Anju *et al.* (2021) [2].

Table 4.3.1: Biochemical parameter of different treatment groups before and after treatment.

Parameters	Groups	Day 0	Day 7	Day 21
Total Protein (g/dl)	A	5.40 ± 0.35	5.08 ± 0.45	5.42 ± 0.82
	B	4.69 ± 0.31	5.21 ± 0.82	5.50 ± 0.35
	C	5.79 ± 0.33	6.06 ± 0.23	5.99 ± 0.33
Albumin (g/dl)	A	2.06 ± 0.16	1.88 ± 0.12	2.42 ± 0.17
	B	1.89 ± 0.12	2.25 ± 0.15	2.22 ± 0.25
	C	2.40 ± 0.15	2.12 ± 0.15	2.41 ± 0.14
Globulin (g/dl)	A	3.34 ± 0.29	3.21 ± 0.40	3.00 ± 0.18
	B	2.79 ± 0.24	2.96 ± 0.15	3.24 ± 0.33
	C	3.38 ± 0.40	3.93 ± 0.25	3.58 ± 0.39
Total Bilirubin (mg/dl)	A	0.41 ± 0.13	0.54 ± 0.13 ^a	0.46 ± 0.08
	B	0.99 ± 0.37	0.30 ± 0.06 ^{ab}	0.29 ± 0.05
	C	0.34 ± 0.10	0.16 ± 0.30 ^b	0.42 ± 0.13
Direct Bilirubin (mg/dl)	A	0.27 ± 0.10	0.26 ± 0.05 ^a	0.23 ± 0.04
	B	0.68 ± 0.24 ^x	0.15 ± 0.02 ^{aby}	0.19 ± 0.03 ^{xy}
	C	0.19 ± 0.07	0.08 ± 0.01 ^b	0.20 ± 0.06
Indirect Bilirubin (mg/dl)	A	0.14 ± 0.03	0.27 ± 0.10	0.23 ± 0.06
	B	0.40 ± 0.18	0.15 ± 0.04	0.10 ± 0.02
	C	0.14 ± 0.05	0.08 ± 0.02	0.22 ± 0.08
Alanine Transferase (IU/L)	A	118.91 ± 20.38	100.95 ± 16.96	102.67 ± 16.24
	B	129.03 ± 21.60	123.17 ± 13.52	112.53 ± 22.30
	C	74.49 ± 16.07	88.57 ± 17.35	90.55 ± 15.82
Aspartate Transferase (IU/L)	A	105.16 ± 19.02	118.43 ± 23.62	101.74 ± 15.39
	B	131.15 ± 25.97	104.77 ± 13.44	87.05 ± 11.51
	C	104.82 ± 18.57	94.31 ± 13.03	85.31 ± 15.37
Blood Urea Nitrogen (mg/dl)	A	18.60 ± 4.66	19.26 ± 3.13	20.82 ± 4.25
	B	31.66 ± 6.66	16.92 ± 2.72	16.66 ± 5.46
	C	20.78 ± 3.89	17.46 ± 2.25	16.14 ± 2.99
Creatinine (mg/dl)	A	0.74 ± 0.15	1.04 ± 0.10 ^a	0.67 ± 0.10
	B	1.16 ± 0.40	0.68 ± 0.06 ^b	0.67 ± 0.07
	C	0.79 ± 0.07	0.93 ± 0.12 ^{ab}	0.95 ± 0.12
Alkaline Phosphatase (IU/L)	A	87.64 ± 7.20	93.34 ± 10.41 ^a	89.20 ± 9.85
	B	98.68 ± 11.93 ^x	57.03 ± 7.76 ^{by}	79.94 ± 7.75 ^{xy}
	C	72.55 ± 6.15	81.23 ± 10.55 ^{ab}	83.38 ± 11.70

Values with different superscript letters (a, b) are significantly different between groups. Values with different superscript letters (x, y) are significantly different within groups ($p < 0.05$).

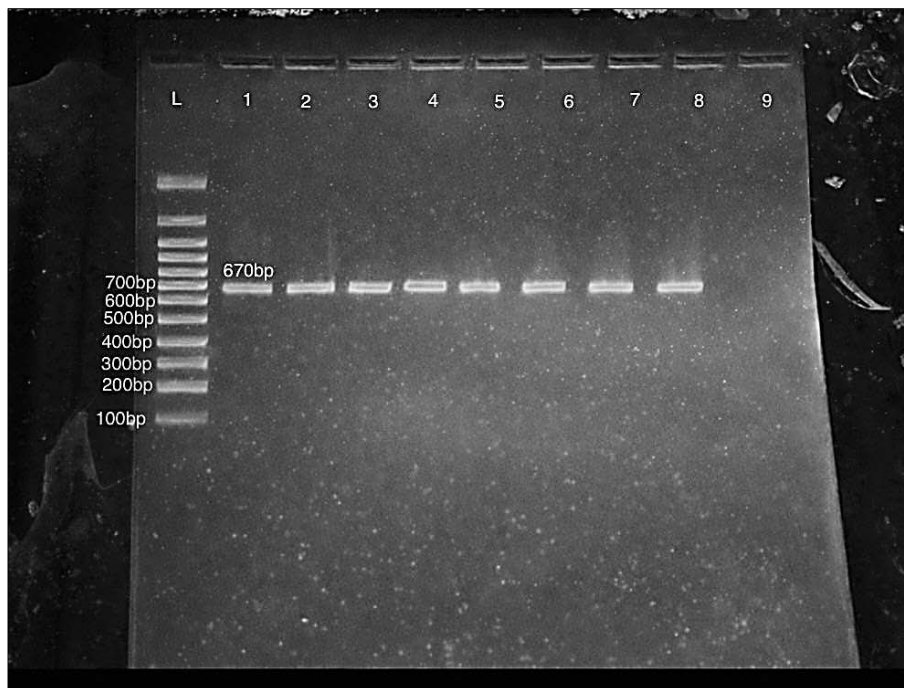
4.4 Evaluation of blood smear examination and polymerase chain reaction

Peripheral blood smear examination showed a progressive decline in *Babesia gibsoni* parasitaemia in all treatment groups. On day 7, smear-based efficacy was 70% in Group A and 80% each in Groups B and C. By day 21, parasitological clearance reached 90% in all groups, with only one dog remaining smear positive in each group. Clinically, Group B showed complete recovery in all dogs, whereas Group C exhibited relapse in 30% of cases despite similar smear clearance.

PCR evaluation revealed lower clearance rates compared to

smear examination, reflecting its greater sensitivity in detecting residual infection. On day 7, PCR-based efficacy was 50% in Group A and 60% in Groups B and C. By day 21, Groups A and B achieved 80% molecular clearance, whereas Group C remained at 60%. These findings indicate superior sustained clinical and molecular efficacy of the combination therapy protocols, particularly in Group B, compared with diminazene aceturate monotherapy.

Smear negativity alone may not indicate complete parasitological cure, and improved PCR clearance with absence of relapse in Group B suggests better control of chronic carrier status.



Lane L: 100bp DNA ladder
Lane 1 to 8: Positive samples
Lane 9: Negative sample

Plate 4.4.1: PCR amplification of *B. gibsoni* using 18s rRNA primers

4.5 Comparative evaluation of therapeutic regimens

A comparison of all three treatment protocols revealed clear differences in outcomes. Group B had the best response, marked by quick clinical improvement, full recovery of all dogs, early normalization of blood parameters, and satisfactory clearance of parasitaemia. Group A also proved effective but required more time to achieve similar results. In contrast, Group C showed lower sustained efficacy, incomplete parasite removal, and higher relapse rates. Regarding blood recovery, Groups A and B displayed a progressive correction of anemia and thrombocytopenia, while Group C improvement was slower. Biochemical recovery was also more consistent in the combination therapy groups. These findings suggest that combination treatments offer broader and more effective with synergistic effect to control the infections and their systemic effects.

5. Conclusion

The present study concludes that adjunct administration of *Crotalus horridus* 200C with doxycycline-enrofloxacin-metronidazole therapy significantly enhanced therapeutic outcomes in dogs naturally infected with *Babesia gibsoni*. The adjunct protocol resulted in faster clinical improvement, superior correction of anemia and thrombocytopenia, improved biochemical recovery, higher molecular clearance, and complete prevention of relapse during the observation period compared with conventional therapy alone and diminazene aceturate monotherapy.

The pronounced recovery of platelet counts highlights the potential supportive role of *Crotalus horridus* in managing hemorrhagic complications and persistent thrombocytopenia associated with canine babesiosis. Therefore, *Crotalus horridus* 200C may be considered a promising adjunct therapeutic option in the clinical management of canine babesiosis, particularly in cases showing severe hematological compromise. Further studies involving larger sample sizes and longer follow-up periods are recommended

to validate its long-term role in parasitological clearance and prevention of carrier states.

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