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Therapeutic evaluation of homoeopathic drug *Crotalus horridus* 200C against Ehrlichiosis-infected dogs in Mizoram

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ABSTRACT

Objective: To study, the effect of a homoeopathic medicine *Crotalus horridus* 200C on ehrlichiosis in dogs in an endemic area of Aizawl district of Mizoram state of India.

Materials and Methods: To evaluate the efficacy of *Crotalus horridus* 200C against ehrlichiosis dogs. 12 positive cases confirmed by polymerase chain reaction (PCR) were divided into two groups comprising six dogs in each group. One group was treated with standard therapy (*doxycycline*) and other group was treated with *Crotalus horridus* 200C at 4 pills orally for 20 days. Clinical improvement of affected dogs was recorded after therapy. Important haemato-biochemical parameters before and after therapy such as haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), differential leukocyte count (DLC), platelet count, total protein, albumin, globulin, A:G ratio, total bilirubin, serum creatinine, blood urea nitrogen (BUN), and liver-specific enzymes namely alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assessed following standard protocol. All the parameters were compared with a control healthy group (T3). All experiment dogs were from different age with different breeds and bloods were collected at forenoon only.

Results: PCR test yielded 13 dogs positive out of 67 suspected samples screened (19.40%) with an amplification of 387 bp fragment from 16S rRNA gene of E. Canis. Off total positive, only 8 (61.53%) could be detected in peripheral blood smear. *Crotalus horridus*-treated group of dogs showed clinical recovery from fever and temperature to normalcy by the 14th day posttreatment. Haemato-biochemical profiles of affected dogs such as Hb, PCV, TEC, TLC, DLC, platelet count, total protein, albumin, globulin, A:G ratio, total bilirubin, serum creatinine, BUN, and liver-specific enzymes namely ALT and ALP were turned to normalcy within 21 days of post-treatment.

Conclusion: Nested PCR assay had been shown to be

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sensitive and specific for detection of Ehrlichia canis. *Crotalus horridus* 200C may be an effective and choice of drug for control of canine ehrlichiosis.

Keywords: *Crotalus horridus* 200C, Ehrlichiosis, Haemato-biochemical changes, Homoeopathy, Veterinary medicine

INTRODUCTION

Canine ehrlichiosis is a tick-borne disease caused by Ehrlichia canis, also known as canine monocytic ehrlichiosis (CME), canine haemorrhagic fever, tracker dog disease, canine tick typhus, Nairobi bleeding disorder, and tropical canine pancytopenia.[1,2] CME is currently reported throughout the world[3] but at higher frequencies in tropical and subtropical regions including Asia, Africa, Europe, America,[4-6] and India.[7,8] The infection is manifested by fever, depression, dyspnea, anorexia, weight loss, limping as well as unwillingness to move due to arthritis, together with pains in musculoskeletal system.[8] Laboratory findings include thrombocytopenia, leukopenia, mild anaemia (normocytic and nonregenerative), and hypergammaglobulinemia. Serum biochemistry of the affected dogs reveals hypoproteinemia, hypoalbuminemia, hyperglobulinemia, increased activities of the alkaline phosphatase (ALP) and alanine aminotransferase (ALT) and the rise of the concentration of creatinine and urea.[9,10]

Diagnosis of ehrlichiosis in dogs is based on the symptoms along with microscopic, haematological, biochemical, serological, and molecular examinations. In infected host, Ehrlichias are found in three forms in peripheral smears creating cytoplasmatic inclusion bodies – initial corpuscles, morulas; formed by aggregation of inclusion bodies and elementary corpuscles; created after the morula's disintegration.[11,12] The most sensitive diagnostic method of ehrlichiosis is the polymerase chain reaction (PCR) technique.[13,14]

Regardless of the species of Ehrlichia or the form of the disease, the choice of drugs to treat ehrlichiosis is tetracycline group of drugs.[15] High use of antibiotics can have negative aspects for animal health, human health, and the environment.[16] This rapid rise in usage of veterinary antibiotics necessitates the development of sustainable alternative; antibiotics are partly replaced by complementary or alternative medicine of which Homoeopathy is the most frequently applied.[17]

Limited reports have been published in homoeopathic and alternative medicine journals on veterinary clinical trials with homoeopathic medicine/drugs where methodological weakness is noticed. Homoeopathic remedies have significant benefits since there are no residues in animal products nor does Homoeopathy generate resistant microorganisms. Homoeopathy aims to activate self-healing mechanisms of the body. In view of the above, the present study was carried out to detect E. canis by molecular technique and find out the therapeutic efficacy of a homoeopathic medicine *Crotatus horridus* against ehrlichiosis which was used against other tick-borne diseases such as babesiosis but not in ehrlichiosis.

MATERIALS AND METHODS

The study was conducted in the Department of Veterinary Medicine, College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl, in between August 2012 and July 2014. The study area lay between 25°6'N and 27°4'N latitude and 93°20'E to 95°15'E longitude.

Sample Size

Clinical cases of ehrlichiosis in dogs showing symptoms such as fever, diarrhoea, staggering gait, anaemia, debilitated condition, and presence of ticks were recorded during the study period. All-together, 67 dogs were initially suspected for ehrlichiosis. Confirmation of ehrlichiosis was based on the presence of E. canis in peripheral blood smear examination and PCR. 13 cases were confirmed by Polymerase chain reaction and out of these 8 also tested positive for E. canis in peripheral blood smear. For randomisation 12 cases were considered. One case with positive PCR and blood smear was excluded from the study for convenience of randomisation and treated in general OPD [Chart 1].

Collection of Blood

About 5 ml blood samples were collected each ailing dog with or without anticoagulant for haemato-biochemical analysis. The blood collected with anticoagulant ethylene diamine tetra acetic

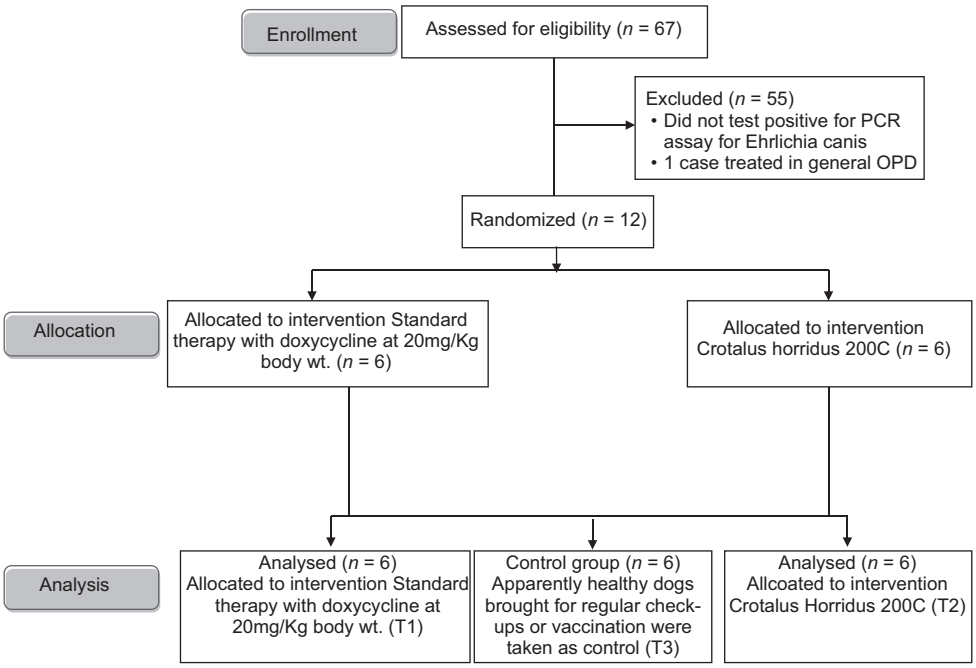

Chart 1: Participant flow diagram

acid was used for PCR and haematology, and blood without anticoagulant was used for serum separation. Collected serum samples were stored in deep freeze at −20°C until used for biochemical and enzymatic estimations. Similarly, a few samples were collected from healthy dogs for comparison. Giemsa-stained smears prepared from the blood were examined microscopically for the presence of E. canis morulae.

Molecular Detection of Ehrlichia canis

Fresh blood samples obtained from dogs were screened for the detection of E. canis DNA using PCR as per standard method.[18]

Extraction of genomic DNA from blood

DNA extraction was carried out using the DNA assay blood and tissue kit (Quiagen® Kit, Catalog No 69504) as per manufacturer's protocol. In brief, about 100 μl anticoagulated blood taken individually from each of the samples in a 2 ml microcentrifuge tube was lysed in 20 μl proteinase K and the final volume adjusted to 220 μl by adding phosphate buffered saline before adding lysis buffer. The tubes were then incubated at 56°C in water bath for 10 min. Samples were then passed through silica gel based spin column and washed 2 times and then finally eluted with 100 μl elution buffer after following all the mid-step protocols, and the templates were kept at −20°C.

Polymerase chain reaction assay for detection of Ehrlichia canis

Target gene (16S rRNA) amplification was done using the nested PCR technique following the method of Murphy *et al.*[18] with modifications. The first PCR amplification aimed at amplifying a 477-bp region of the 16S rRNA gene of *Ehrlichia* species. Oligonucleotides ECC (5′AGAACGAACGCTGGCGGCAAGC3′) and ECB (5′CGTATTACCGCGGCTGCTGGCA3′) were used as sense and antisense primers, respectively for outer region. The nested PCR amplification aimed at amplifying 387-bp internal fragment specific to E. canis. Oligonucleotides ECA (5′CAATTATTTATAGC CTCTGGCTATAGGA 3′) and HE3 (5′ TATAGGTACCGTCATTATCTTCCCTAT 3′) were used as sense and antisense primers, respectively. Reaction mixture of 25 μl volume contains 2.5 μl of 10X PCR buffer, 0.5 μl of dNTP (10 mM each), forward and reverse primer 1 μl (10 pmol) each, Taq polymerase 0.2 μl (5 U/μl), template DNA 5 μl, and nuclease free water adjusted to 25 μl. A hot start PCR was used with initial denaturation of 94°C for 5 min and then 30 cycles of 94°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 2 min. A final extension was kept at 72°C for 7 min for primary amplification. Nested PCR was performed where 2 μl of primary product

was taken as template and annealing temperature was set at 55°C keeping other conditions as such. The PCR product was subjected to electrophoresis in an agarose gel (1.5%) prestained with ethidium bromide (0.5 μg/ml), and subsequent visualization was done in gel documentation system (alpha image system).

Evaluation of Therapeutic Potential of Homoeopathic Medicine Against Ehrlichiosis in Dogs

Twelve dogs positive for ehrlichiosis were divided randomly into two groups ($n = 6$) namely T1 and T2. Six apparently healthy dogs brought for regular check-up or vaccination were taken as control group (T3).

Dogs of T1 were treated with standard therapy with *doxycycline* at 20 mg/kg body weight orally for 20 days along with supportive therapy and T2 was treated with a Homoeopathy drug *Crotalus horridus* 200C (Hylands, USA) at 4 pills orally for 20 days. Therapeutic potency was evaluated by improvement of clinical signs, haemato-biochemical parameters, blood smear, and PCR assay for E. canis infection on days 0, 7, 14, and 21 posttreatment.

Clinical examination of dogs infected with ehrlichiosis before and after therapy

Each dog was subjected to detailed clinical examination as per standard procedure.[19] The presence of symptoms/signs/manifestations and involvement of different body system and systemic states were recorded on days 0, 7, 14, and 21 posttreatment.

Blood sample collection

To access the therapeutic efficacy, blood samples were collected on days 0, 7, 14, and 21 posttreatment by the same procedure as described elsewhere.

PCR was carried out as described elsewhere on days 0, 7, 14, and 21 blood samples to confirm the duration of presence of E. canis in blood and the efficacy of the drug in trial from the day of the starting of the treatment in different group.

Haemato-biochemical investigation

Haematological assays were performed within 2 h of blood collection by using an automated cell counter (Minos ST; Vet, Montpelier, France) calibrated for canine blood. The parameters measured were hematocrit, haemoglobin (Hb) concentration, total erythrocyte count, total leukocyte count (TLC), platelet count, and differential leukocytic count.

The total protein and albumin were estimated by the Biuret method[20] whereas globulin was estimated by calculation. The concentration of ALT was determined spectrophotometrically using the method of Reitman and Frankel.[21] The level of ALP in the serum was determined as described by Omotainse *et al.*,[22] using spectrophotometry method. Creatinine (alkaline picrate method), blood urea nitrogen (BUN) (Mod. Berthelot method), total bilirubin, and direct bilirubin (modified Jendrasik and Grof method) were also estimated by using spectrophotometry method.

Haemato-biochemical analyses were carried out on days 0, 7, 14, and 21 to know the efficacy of the therapy.

Therapeutic Evaluation

Therapeutic evaluation was done on the basis of improvement of clinical signs, blood haematology, and biochemical changes and negative results of PCR.

Statistical Analysis

Data were analyzed by using two-way ANOVA by Statistical Package SPSS 16 (SPSS, Science, Chicago, USA). Differences were considered statistically significant at $P < 0.05$ level.

RESULTS

During the study period, 67 dogs were suspected for ehrlichiosis, of which 8 cases were confirmed by blood smear (11.94%) whereas 13 cases were confirmed by PCR (19.40%).

Molecular Detection of Ehrlichia canis by Nested Polymerase Chain Reaction

Nested PCR was performed by using genus-specific external primer (477 bp) and species-specific nested internal primer (387 bp) to detect E. canis organism. Nested PCR positive samples were demonstrated which yielded an amplification of 387 bp fragment from 16S rRNA gene of E. canis [Figure 1a and b].

Effect of Therapy on Clinical Signs and Physical Parameters

Physical parameters improved after 14 days in both the treatment groups [Table 1]. There was significant reduction ($P < 0.05$) of temperature, pulse, and respiration on day 7 posttherapy in both the groups. The clinical signs [Table 1a] were also improved after treatment in both the groups. All the ailing animals showed alertness and improved

Table 1: Efficacy of *doxycycline* (T1) and *Crotalus horridus* (T2) on physical parameters of *Ehrlichia*-infected dog

Parameters	Group (*n*=6)	Days			
		0	7	14	21
Temperature (°F)	T1	$103\pm0.16^{Aa**}$	$101.3\pm0.16^{bA*}$	$101\pm0.21^{bcBC*}$	$101\pm0.15^{bcd*}$
	T2	$104\pm0.20^{ABa**}$	102.5 ± 0.23^{ab}	101.5 ± 0.16^{abc}	101.5 ± 0.12^{abcd}
	T3#	101.28 ± 0.12^{C}	101.28±0.12	101.28±0.12	101.28±0.12
Pulse (per/min)	T1	$88.5\pm0.82^{aA**}$	80.5 ± 1.94^{ab}	$74.5\pm1.86^{bc*}$	72.5 ± 0.30^{cd}
	T2	$86.5\pm0.70^{aAB**}$	83.5 ± 0.65^{ab}	$79\pm0.67^{bc*}$	74 ± 0.40^{cd}
	T3#	72.33 ± 0.98^{C}	72.33±0.98	72.33±0.98	72.33±0.98
Respiration (per/min)	T1	45.5±0.85	40.5±0.85	35.5±0.50	35.5±0.50
	T2	39±0.38	37±0.80	36±0.79	34.5±0.62
	T3#	33.5±0.88	33.5±0.88	33.5±0.88	33.5±0.88

*Significant difference (P<0.05); **Significant difference (P<0.01). Values bearing different superscript small letter in column wise and capital letter in row wise showed significant difference. #T3 stands for healthy group

Table 1a: Efficacy of *doxycycline* (T1) and *Crotalus horridus* (T2) on *Ehrlichia*-infected dogs on clinical improvement

Parameters	Group (*n*=6)	Days			
		0	7	14	21
Physical parameters	T1	+	−	−	−
	T2	+	−	−	−
Clinical signs	T1	+	+	−	−
	T2	+	+	−	−
Presence (+) or absence (−) of Ehrlichia canis DNA in blood	T1	+	+	−	−
	T2	+	+	+	−

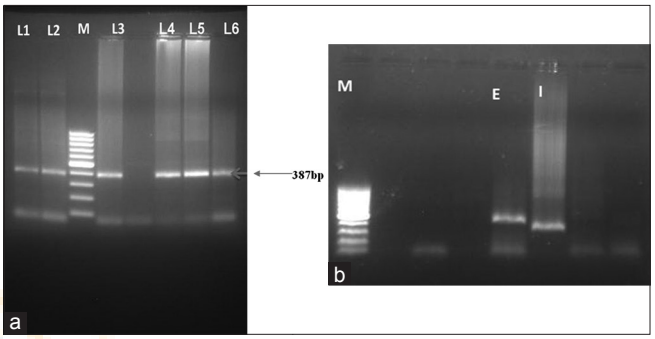

Figure 1: (a) Gel photo showing amplification of target gene of Ehrlichia canis. Lane 1–6: Positive for Ehrlichia canis; Lane M: 100bpDNA ladder; Lane N: Negative sample. (b) Gel picture of Ehrlichia canis nPCR product. M: 100bp DNA ladder; E: External product (477bp); I: Internal nested product (387bp)

appetite. PCR assay showed negative result in T2 group on day 21 posttherapy whereas no samples were detected positive by PCR assay in T1 group on day 14 posttherapy [Table 1a]. The critical analysis revealed that the *Crotalus Horridus* had almost equal intensity with doxycycline to manage the ehrlichiosis infection.

Effect of Therapy on Haematological Profile

The haematological profiles of dogs (before and after therapy) are shown in Table 2. The Hb, packed cell volume, total erythrocyte count, TLC, and platelet count was significantly improved ($P < 0.01$) on day 21 in both T1 and T2 groups after therapy. The results clearly indicated that both *doxycycline* and *Crotalus horridus* 200C therapy improved the hematological values in ehrlichiosis-infected dogs.

Changes in monocyte count are significantly ($P < 0.01$) reduced in T1 group of dogs receiving *doxycycline* as a standard therapy. However, significant changes were also found in T2 group of dogs receiving *Crotalus horridus* 200C as experimental therapy in a lesser intensity. Neutrophils, lymphocytes, and eosinophil counts were also found improved after 21 days therapy in both the groups of dogs.

Effect of Therapy on Serum-biochemical Profile

Mean ± standard error of serum biochemical profiles of dogs (before and after therapy) has been shown in Table 3. Total protein concentration of T1 and T2 was significantly decreased ($P < 0.05$) on day 0 as compared to healthy dogs (T3). However, protein values were increased in both the treated groups after therapy. Albumin level was significantly ($P < 0.05$) increased in T1 and T2 from day 0 (1.43 ± 0.35 g/dl and 1.17 ± 0.19 g/dl) to day 21 (3.78 ± 0.19 g/dl and 3.09 ± 0.28 g/dl) and there was no significant difference in globulin level in both the T1 and T2 before and after therapy. The levels of creatinine and BUN did not differ significantly from healthy group and nonsignificant decrease in both the parameters by the end of therapy depicted the therapeutic efficacy.

There was significant increase ($P < 0.05$) in the value of ALT and ALP on day 0 of both the treated groups (T1

Table 2: Evaluation of hematological values on clinical trial between *doxycycline* (T1) and *Crotalus horridus* (T2) on *Ehrlichia*-infected dogs

Parameters	Group (*n*=6)	Mean±SE Days			
		0	7	14	21
Hb (g/dl)	T1	$7.28\pm1.71^{Aa**}$	$7.95\pm1.20^{Aab**}$	$9.81\pm0.98^{Abc**}$	$10.25\pm0.29^{Ac*}$
	T2	$7.15\pm01.41^{Aba**}$	$7.76\pm3.11^{ABab**}$	$9.15\pm0.88^{ABb**}$	$10.01\pm0.29^{ABb*}$
	T3	11.7 ± 0.31^{C}	10.7 ± 0.30^{C}	11.9 ± 0.24^{ABC}	10.8 ± 0.33^{ABC}
PCV (%)	T1	$22.5\pm4.32^{Aa**}$	$24.33\pm4.22^{Aab**}$	$30.33\pm1.0^{Abc**}$	$33.66\pm1.36^{Acd**}$
	T2	$22.16\pm3.31^{Aba**}$	$23.46\pm3.38^{ABab**}$	$25.90\pm3.85^{ABabc**}$	$27.87\pm1.19^{ABcd**}$
	T3	$34.16\pm0.60^{C**}$	$34.06\pm0.61^{C**}$	$33.16\pm0.67^{C**}$	$32.16\pm0.64^{ABC**}$
TEC ($\times10^6$/µl)	T1	$3.25\pm0.92^{Aa**}$	$4.15\pm0.79^{Aab**}$	$4.75\pm0.52^{Aabc*}$	5.27 ± 0.59^{cd}
	T2	$3.13\pm0.48^{Aba**}$	$3.43\pm0.43^{ABab**}$	$3.89\pm0.56^{ABbc*}$	4.34 ± 0.25^{cd}
	T3	$4.96\pm0.13^{C**}$	$4.56\pm0.12^{ABC**}$	$4.36\pm0.15^{ABC*}$	4.66 ± 0.10
TLC ($\times10^3$/µl)	T1	$58.83\pm21.95^{Aa*}$	67.16 ± 2.27^{Aab}	83.00 ± 13.20^{abc}	101.98 ± 14.17^{cd}
	T2	$82.50\pm16.69^{AB*}$	83.25 ± 16.71^{AB}	85.63 ± 34.99	88.56 ± 20.09
	T3	$103.8\pm0.45^{BC*}$	$102.8\pm0.47^{BC*}$	105.8 ± 0.47	101.8 ± 0.49
Platelets ($\times10^3$/µl)	T1	$85.96\pm16.44^{Aa**}$	$105.37\pm44.13^{Aab**}$	$110.16\pm44.36^{Abc**}$	$121.50\pm46.18^{Acd**}$
	T2	$88.77\pm77^{ABa**}$	$91.25\pm13.01^{ABab**}$	$98.25\pm19.64^{ABabc**}$	$119.84\pm20.19^{ABbcd**}$
	T3	$267.57\pm16.64^{C**}$	$260.57\pm15.64^{C**}$	$257.57\pm11.64^{C**}$	$247.57\pm13.64^{C**}$
Neutrophil (%)	T1	67.33 ± 2.62	68.33 ± 2.40	$67.5\pm2.51^{A*}$	$67.33\pm2.62^{A**}$
	T2	74.33 ± 1.49	74.66 ± 1.20	$77\pm0.81^{B*}$	$79.33\pm0.80^{B**}$
	T3	76.5 ± 1.47	77.5 ± 1.42	$79.5\pm1.40^{BC*}$	$72.5\pm1.49^{BC**}$
Lymphocyte (%)	T1	21.5 ± 2.17	22.16 ± 1.92	21.66 ± 2.31	21.5 ± 2.17
	T2	15.66 ± 1.64	15.50 ± 1.62	11.56 ± 1.44	14.5 ± 0.71
	T3	21.33 ± 1.74	21.03 ± 1.77	22.30 ± 1.72	21.63 ± 1.74
Monocyte (%)	T1	$7.66\pm0.96^{Aa**}$	$5.33\pm0.80^{Aab**}$	$2.33\pm0.33^{Ac*}$	1.5 ± 0.22^{cd}
	T2	$6.66\pm0.42^{AB**}$	$5.16\pm0.65^{AB**}$	$4.83\pm0.60^{B*}$	3.50 ± 0.61
	T3	$2.16\pm0.47^{C**}$	$2.06\pm0.45^{C**}$	$2.00\pm0.47^{AC*}$	2.076 ± 0.46
Eosinophil (%)	T1	$1.5\pm0.22^{A**}$	$1.66\pm0.33^{A**}$	$1.50\pm0.22^{A**}$	1.50 ± 0.22^{A}
	T2	$3.3\pm0.42^{AB**}$	$3.33\pm0.43^{AB**}$	$3.5\pm0.56^{AB**}$	1.83 ± 0.30^{AB}
	T3	$0.66\pm0.21^{C**}$	$0.69\pm0.22^{C**}$	$0.77\pm0.20^{C**}$	0.76 ± 0.22^{C}

*Significant difference (P<0.05); **Significant difference (P<0.01). Values bearing different superscript small letter in column wise and capital letter in row wise showed significant difference. SE: Standard error; PCV: Packed cell volume; TLC: Total leukocyte count; TEC: Transluminal endarterectomy catheter; Hb: Haemoglobin

and T2) as compared to healthy group (T3). There was significant ($P < 0.05$) decrease in the values of ALT and ALP on day 21 in both the treated groups. Dogs of both groups are depicting steady improvement in the ALT and ALP values as compared to healthy one. Direct bilirubin and total bilirubin also reveal steady nonsignificant improvement on both the groups of dogs. Critical analysis of the result on liver enzyme profile revealed steady improvement on the standard and experimental therapy.

DISCUSSION

In the present investigation, of 67 suspected dogs for canine ehrlichiosis, 13 (19.40%) were found positive by PCR. In the past, various studies have been carried out regarding the molecular prevalence of E. canis worldwide and the prevalence rate has been reported ranging from 3.1% to 88.0%.[23] As regards to the Indian scenario, the prevalence of E. canis varied from 19% to 59%.[24-27] The variation in the prevalence reported by various workers may be attributed to sample size, geographical area, and climatic conditions which directly influence the tick population and time of sample collection.

The 16S rRNA gene fragment that was amplified in this study was also explored by Iqbal *et al.*,[28] McBride *et al.*,[29] Inokuma *et al.*,[30] and Unver *et al.*[6] with reasonably reliable results. To date, the epidemiological studies of canine ehrlichiosis in India have been based on blood smear results, as well as on serological evidence of circulating antibodies. The enhanced sensitivity of nested PCR for the detection of canine ehrlichiosis was earlier reported by Warner and Dawson[31] and Egenvall

Table 3: Evaluation of serum biochemical parameters on clinical trial between *doxycycline* (T1) and *Crotalus horridus* (T2) on Ehrlichia-infected dogs

Parameters	Group (*n*=6)	Mean±SE Days			
		0	7	14	21
Total protein (g/dl)	T1	5.09±0.27^{A**}	5.34±0.24^{A**}	6.09±0.32^{A**}	6.72±0.43
	T2	5.43±0.36^{AB**}	5.47±0.37^{AB**}	5.73±0.41^{B**}	6.32±0.48
	T3	7.19±0.11^{C**}	7.09±0.12^{C**}	7.11±0.11^{AC**}	7.31±0.11
Albumin (g/dl)	T1	1.43±0.35^{Aa**}	2.33±0.19^{Ab**}	2.65±0.08^{Abc**}	3.78±0.19^{Ad*}
	T2	1.17±0.19^{ABa**}	1.26±0.20^{ABab**}	1.88±0.20^{ABabc**}	3.09±0.28^{ABd*}
	T3	4.10±0.25^{C**}	4.22±0.27^{C**}	4.15±0.22^{C**}	4.07±0.25^{C*}
Globulin (g/dl)	T1	1.10±0.11^{A*}	3.01±0.27^{A**}	3.45±0.38	3.99±0.26
	T2	4.26±0.31^{B**}	4.07±0.26^{B**}	3.87±0.24	3.25±0.37
	T3	3.09±0.22^{BC**}	3.11±0.24^{AC**}	3.19±0.25	3.22±0.20
A: G	T1	0.42±3.79^{Aa**}	0.82±0.12^{Aab**}	0.83±0.11^{Aabc*}	0.96±0.0.07^{bcd}
	T2	0.28±0.06^{ABa**}	0.31±0.06^{ABab**}	0.48±0.04^{ABabc*}	1.03±0.19^{cd}
	T3	1.40±0.20^{C**}	1.42±0.22^{C**}	1.39±0.20^{C*}	1.45±0.21
BUN (g/dl)	T1	25.83±0.16	25.17±4.25	24.67±4.35	23.17±3.70
	T2	26.67±4.64	25.50±4.74	24.67±3.83	26.67±4.64
	T3	34.45±8.35	34.45±8.35	34.45±8.35	34.45±8.35
Creatinine (mg/dl)	T1	1.16±0.18	0.95±0.08	0.92±0.08	0.76±0.22
	T2	1.10±0.18	1.08±0.11	0.98±0.07	0.82±0.08
	T3	0.62±0.15	0.66±0.15	0.67±0.15	0.62±0.15
ALT (µ/L)	T1	141.10±50.65	129.17±36.30	105.83±21.18	77.17±4.43^{A*}
	T2	157.81±50.65	140.91±41.93	125.83±18.07	92±3.28^{AB*}
	T3	43.79±8.77	43.99±8.73	42.79±7.73	44.79±8.76^{C*}
ALP (µ/L)	T1	205.81±29.34	138.50±15.25	128.50±15.25	93.17±6.88
	T2	195.01±32.14	141.50±14.16	132.67±15.25	111.33±12.61
	T3	42.48±9.40	42.58±9.40	43.48±8.40	42.08±8.45
Total bilirubin (mg/dl)	T1	1.81±0.39	1.33±0.28	0.98±0.15	0.75±0.04
	T2	1.84±0.39	1.53±0.28	1.09±0.19	0.96±0.04
	T3	0.67±0.21	0.63±0.25	0.69±0.21	0.62±0.24
Direct bilirubin (mg/dl)	T1	0.88±0.10	0.78±0.08	0.63±0.07	0.64±0.07
	T2	1.33±0.04	0.81±0.10	0.60±0.07	0.58±0.08
	T3	0.55±0.22	0.58±0.21	0.65±0.22	0.52±0.25

*Significant difference (P<0.05); **Significant difference (P<0.01). Values bearing different superscript small letter in column wise and capital letter in row wise showed significant difference. SE: Standard error; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen

et al.[32] Many authors already described the superior sensitivity and specificity of PCR in diagnosing ehrlichiosis when compared to serology.[28,33] In the present study, dogs were found to be positive for E. canis by nested PCR after 7 days of *doxycycline* as well as *Crotalus horridus* treatment, indicating the persistence of infection. It may also be an indicator of the short duration of treatment, especially in immune-compromised dogs. Hence, it is a highly effective tool for assessing the clearance of organisms after therapy.

Oral *doxycycline* has long been the drug of choice for treatment of CME. Failure to clear infections from hosts that respond to treatments for ehrlichial diseases can result in asymptomatic carriers that remain sources of infection for vectors and naive vertebrate hosts, so an alternative treatment regimen should be evaluated. Moreover, prolonged use of antibiotic therapy is associated with greater risk and likelihood of developing drug resistance. This phenomenon is a huge global concern and is mostly the result of history of indiscriminate overuse of these agents in both medicine and agriculture. The issue of resistance, with the subsequent development of recurrent infections, chronic infections, and the proliferation of progressively stronger strains of microorganisms has been the focus of extensive investigation and concern. There is definite evidence that this agent actually

delays the development of host immunity while promoting a state of perpetual illness due to multiple factors associated with their use.[34] Homoeopathy has been used in animals to treat a multitude of conditions.[35] Our findings regarding clinical recovery of the dogs from ehrlichiosis with *Crotalus horridus* is encouraging. On the clinical score, *Crotalus horridus* and doxycycline showed progressive and significant regression in clinical signs on days 7, 14, and 21 posttherapy and improvement of haemato-biochemical values of infected dogs and there was no significant difference between the groups. Similarly, there was progressive decline in the parasitized erythrocytes in both the groups. However, reduction in parasitized erythrocytes in case of *Crotalus horridus* was evident only on day 21, but parasitized erythrocytes reduced significantly from the 7th day onward in case of *doxycycline*. The effectiveness of *Crotalus horridus* has been described in babesiosis of cattle and dog,[35,36] on fever, hemorrhagic diathesis, yellow color of conjunctiva, jaundice, purpura hemorrhagica, profound anaemia;[37] however, it has never been evaluated in canine ehrlichiosis. The results showed an encouraging response of *Crotalus horridus* in the management of canine ehrlichiosis. The clinical efficacy of the homoeopathic drug was comparable with modern allopathic drug doxycycline. Moreover, the use of homoeopathic drugs in the management of ehrlichiosis in dogs has not previously been reported.

Our findings regarding clinical recovery of the dogs with ehrlichiosis with *Crotalus horridus* are encouraging. There was a significant reduction ($P < 0.05$) of temperature, pulse, and respiration on the 7th-day posttherapy. The clinical signs were also improved after treatment. All the ailing animals showed alertness and improved appetite. Haemato-biochemical changes due to ehrlichiosis-infected dogs returned to normalcy after *Crotalus horridus* treatment. From the present study, it seems that *Crotalus horridus* can be an effective and alternative in case of ehrlichiosis.

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Conflicts of Interest

There are no conflicts of interest.

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मिजोरम में इहरलिकियोसिस संक्रमित कुत्तों मे होम्योपैथिक दवा क्रोटैलस हॉरिड्स 200सी का चिकित्सकीय मूल्यांकन

सार –

उद्देश्य– प्रस्तुत अध्ययन भारत के मिजोरम प्रान्त में स्थित आइजोल जिले के स्थानिक क्षेत्र में इहरलिकियोसिस संक्रमित कुत्तों मे होम्योपैथिक दवा क्रोटैलस हॉरिड्स 200सी के प्रभाव का अध्ययन करना।

आवश्यक सामग्रियाँ और विधियां– इहरलिकियोसिस संक्रमित कुत्तों में होम्योपैथिक दवा क्रोटैलस हॉरिड्स 200सी के चिकित्सीय प्रभाव का मूल्यांकन करने हेतु, पीसीआर विधि से पुष्टि किये गये बारह धनात्मक प्रकरणों को दो समूहों में प्रति समूह 06 (छह) कुत्तों के हिसाब से बांटा गया। एक समूह मानक चिकित्सा (डाक्सीसाईक्लिन) और दूसरे समूह को 20 दिनों के लिए होम्योपैथिक दवा क्रोटैलस हॉरिड्स 200सी की 4 गोलियां प्रतिदिन मौखिक रूप से देने के बाद प्रभावित् कुत्तों में नैदानिक सुधार अंकित किया गया। चिकित्सा के पूर्व तथा पश्चात महत्वपूर्ण रक्त–जैव रासायनिक मापदंडों जैसे – हीमोग्लोबिन, पीसीवी, टीईसी, टीएलसी, डीएलसी, प्लेटलेट काउंट, टोटल प्रोटीन, एल्बुमिन, ग्लोब्युलिन, एरूजी अनुपात, टोटल बिलीरूबिन, सीरम क्रिएटिनिन,बी यू एन और लिवर के विशेष एन्जाइम्स जैसे–एलनिन अमीनो ट्रांसफेरेज (एएलटी) और क्षारीय फॉस्फेट (एपी) का मापन मानक स्तरों के प्रोटोकॉल द्वारा किया गया। सभी मापदंडों की तुलना नियंत्रित स्वस्थ समूह (टी 3) से की गई। सभी परीक्षणों में कुत्ते भिन्न आयु वर्ग एवम् भिन्न–भिन्न प्रजातियों के थे। रक्तपरीक्षण हेतु रक्त का संकलन सिर्फ मध्यान्ह पूर्व किया गया था।

ई. कार्निस से जीन के 16 तत्छ 387 इंच हिस्से के आवर्धन से 67 सन्दिग्ध नमूने जिन्हें जाँच हेतु लिया गया (19.40 प्रतिशत) में से पीसीआर परीक्षण में 13 कुत्तों को प्रभावित पाया गया। पूरे प्रभावित प्रकरणों में से, सिर्फ 8 (61.53 प्रतिशत) के परिसंचरणीय रक्त में इनकी उपस्थिति पायी गयी। चिकित्सा के 14वें दिन होते–होते सी हारीड्स द्वारा चिकित्सित कुत्तों के समूह में बुखार नैदानिक रूप से ठीक हो गया और शरीर का तापमान सामान्य अवस्था में आ गया। चिकित्सा के 21वें दिन प्रभावित कुत्तों के रक्त–जैव रसायन घटक जैसे– एचबी, पीसीवी, टीईसी, टीएलसी, डीएलसी, प्लेटलेट काउंट, टोटल प्रोटीन, एल्बुमिन, ग्लोब्युलिन, एरूजी अनुपात, टोटल बिलीरूबिन, सीरम क्रिएटिनिन,बी यू एन और लिवर के विशेष एन्जाइम्स जैसे– एलनिन अमीनो ट्रांसफेरेज (एएलटी) और क्षारीय फॉस्फेट सामान्य स्तर पर पहुँच गये।

निष्कर्ष– नेस्टेड पीसीआर परख द्वारा यह दिखाया गया है कि यह ई केनिस के प्रति संवेदनशील है और इसका पता लगाने के लिये विशिष्ट है। सी हॉरिड्स 200 केनाइन इहरलिकियोसिस के नियंत्रण हेतु प्रभावी एवम् पसंदीदा दवा हो सकती है।

Evaluación terapéutica del medicamento homeopático, *Crotalus horridus 200C* en perros con ehrlichiosis en Mizoram

RESUMEN

Objetivos: Estudiar el efecto de un medicamento homeopático Crotalus horridus 200C en la ehrlichiosis en perros en un área endémica de distrito del estado de Mizoram Aizawl de la India. Las terapias alternativas basadas en homeopatía pueden ser eficaces en mejorar estas enfermedades caninas, causadas por garrapatas. En este estudio, se ha investigdo el efecto del medicamento homeopático *Crotalus horridus 200c* en la ehrlichiosis canina en una región endémica del distrito de Aizawl del estado Mizoram de la India.

Materiales y métodos: Para evaluar la eficacia de *Crotalus Horridus 200c* en perros con ehrlichiosis, 12 casos positivos (confirmados por PCR) fueron divididos en dos grupos de 6 perros cada uno. Un grupo fue tratado con la terapia estándar (doxiciclina), mientras que el otro recibió *Crotalus Horridus 200c*, 4 píldoras orales durante 20 días. Después del tratamiento, se registró una mejoría clínica de los perros afectados. Conforme al protocolo estándar, se evaluaron los parámetros hematobioquímicos importantes antes y después del tratamiento, como Hb, hematocrito, recuento total de glóbulos rojos, recuento total y diferencial de leucocitos, recuento plaquetario, proteínas totales, albúmina, globulina, relación A:G, bilirrubina total, creatinina sérica, BUN y enzimas hepáticas específicas (alaninoaminotransferasa (ALT) y fosfatasa alcalina (FA)). Todos los parámetros se compararon con un grupo de control sano (T3) Todos los perros del experimento eran de diferentes edades y razas diferentes y se recogieron muestras de sangre a media mañana solamente.

Resultados: De las 67 muestras sospechosas examinadas, la prueba de la PCR dio lugar a 13 perros positivos (19,40%) con una ampliación del fragmento 387bp del gen 16S rRNA de E. canis. Únicamente en 8 (61,53%) del total de positivos se pudo detectar en el frotis de sangre periférica. El grupo de perros tratados con *Crotalus horridus* mostró una recuperación clínica de la fiebre, y la temperatura se normalizó a los 14 días postratamiento. Los perfiles hemato-bioquímicos de los perros afectados como Hb, hematocrito, recuento total de glóbulos rojos, recuento total y diferencial de leucocitos, recuento plaquetario, proteínas totales, albúmina, globulina, relación A:G, bilirrubina total, creatinina sérica, BUN y enzimas hepáticas específicas (alaninoaminotransferasa (ALT) y fosfatasa alcalina (FA) mostraron valores normales en 21 días después del tratamiento.

Conclusiones: El presente estudio ha reestablecido que la ehrlichiosis es endémica en Mizoram. El ensayo de la PCR anidada ha mostrado ser sensible y específico para la detección de E.canis.El medicamento homeopático *Crotalus horridus* 200 puede ser un remedio de elección eficaz en el control de la ehrlichiosis canina.