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# Subcutaneous ivermectin administration in dogs reduces the risk of heartworm disease in endemic areas

**Manisha Das[1], R. C. Patra[1,*], Rajasri Sahoo[2], Mirashree Pati[1]**

[1] Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar-751003, Odisha, India

[2] Kamal Nehru Women's College, Rama Devi Women's University, Bhubaneswar, India

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## Abstract

The current study aimed to assess the prevalence of heartworm microfilaria in the blood samples collected from dogs, presented to the Referral Veterinary healthcare institutions in Bhubaneswar city, and to compare the efficacy of heartworm preventives, namely ivermectin through the subcutaneous route, and dermal application of selamectin, in reducing the infection risk in endemic areas. Sixty-five dogs suspected of having microfilaria in their blood, based on clinical signs, were screened for confirmation. Forty-two dogs were found positive by wet blood smear examination. The comparative microfilariacidal efficacy of ivermectin at 50g/µg body weight subcutaneously or selamectin at 10mg/kg body weight as a topical application in reducing transmission risk and public health concern in heartworm endemic areas was assessed by treating randomly selected 10 positive dogs, divided equally into two groups, II and III, respectively. Five microfilaria-negative dogs served as controls (Group I). The microfilaria load in the blood, counted by methylene blue staining, declined significantly by day 7 following treatment with ivermectin (Hitek$^{TM}$ from Virbac Animal Health India), whereas the decline in group III after selamectin (Revolution $^{TM}$ from Pfizer Animal Health Division, New York) dermal application was non-significant ($p \geq 0.05$). There was a significant ($p < 0.05$) reduction in total leukocytic counts (TLC) in group II on the 7$^{th}$ and 14$^{th}$ day of treatment, associated with an increase in hemoglobin and PCV. The level of blood urea nitrogen (BUN) and creatinine in group III dogs remained significantly higher than that of the control dogs.

**Keywords:** dirofilariasis; ivermectin; selamectin; dogs; microfilaria

## 1. Introduction

*Dirofilaria immitis* primarily affects the heart and lungs. The infection is characterized by severe cardiac signs of right-sided heart failure and death in untreated cases [1]. Dirofilariasis is a mosquito-transmitted re-emerging zoonosis. The dirofilarial species is considered the most important pathogenic heartworm nematode of dogs. Among those, *D. immitis* is the most dreadful species. The causative parasite is commonly found in dogs, cats, foxes, bears, wolves, and horses, and rarely, in human beings, and the latter is considered the dead-end host, and is associated with pulmonary nodules [2]. *Dirofilaria immitis, Dirofilaria repens, Acanthocheilonema dracunculoides, Acanthocheilonema reconditum, Brugia malayi, Brugia ceylonesis, and Brugia pahangi* are the commonly reported filarial species of dogs. *Dirofilaria repens* localizes in

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subcutaneous tissues in dogs, and infects human beings inadvertently, causing subcutaneous dirofilariasis. The adult worms localize in the right auricle and pulmonary artery, and this association is linked to the manifestation of clinical signs, referable to the cardiovascular system, which are the common manifestations in affected dogs [3]. The mechanical obstructions to the blood flow by the localized adult parasites, and the extent of damage to pulmonary arteries, are factors in the disease severity. The associated clinical signs include dyspnea, coughing, hemoptysis, and increased exercise intolerance [4]. The coughing is induced by immune-mediated pulmonary infiltration with eosinophils. The accompanying right-sided heart failure leads to passive chronic hepatic congestion, resulting in ascites and pleural effusions [5]. The heartworm disease may involve dysfunction of multiple organs, such as lungs, heart, liver, and kidneys [6]. The antigen-antibody reactions may cause arteritis and kidney diseases. The emboli may inflict lesions in lungs, kidneys and brain [5-8]. The disease is characterized by macrocytic hypochromic regenerative anaemia, with a reduced erythrocyte count, hemoglobin, and packed cell volume (PCV), increased total and differential leucocytic counts, and fast erythrocyte sedimentation rate [4, 9]. These manifestations are poorly linked to the presence of microfilaria or larvae of the parasite in the blood circulation. However, their presence helps in making a diagnosis [10]. The adult worms may not always be parasitized for microfilariae which are present in the peripheral blood circulation.

A postmortem survey of indigenous dogs in Odisha established infections with *D. immitis* and *D. repens* in 57% and 14% of carcasses, respectively [11]. The disease has a global distribution, and it is naturally more prevalent in tropical than temperate regions. It is endemic in six continents [7,12, 13, 14]. The northeastern states of India, particularly Assam and Mizoram, are known as hotspots for dirofilariasis [14]. The parasite has also been reported from other areas of the country, including southern India. The arthropod vectors play a crucial role in transmitting microfilaria during blood feeding that follows localization in new hosts in endemic areas. The transmission of *D. immitis* is influenced by several factors, such as climate, availability of host, and vector mosquito population, as mosquitoes play a significant role in the spread of dirofilariasis [15]. Therefore, the geographical distribution and transmission of the parasite are influenced by the environmental factors such as regional temperature, rainfall, and humidity, which influence the vector population and their activity [16]. Hence, climate changes significantly affect the spread of the heartworm parasite. An increase in the mosquito population shortens the duration of extrinsic growth of infective stages and lengthens the season duration for transmission [13].

The treatment of mature heartworm is troublesome for pet owners, dogs, and treating veterinarians, due to the high treatment cost, associated risk, side effects, and exercise intolerance [17]. The control of the disease depends on the frequent ad hoc use of anthelmintics to prevent the disease in definitive hosts [1]. A strategic treatment based on disease epidemiology is practically impossible due to a lack of careful scheduling, depending on parasite dynamics across the seasons [18, 19].

The introduction of macrolide agents and other microfilaricides such as ivermectin, moxidectin, doramectin, milbemycin oxime, and selamectin in different formulations and their use as effective heartworm preventives and therapeutics, has assisted in controlling dirofilariasis. Ivermectin administered orally at 50µg/kg body weight, as a single dose is an excellent microfilaricidal against *D. immitis* [20, 21]. Selamectin is a semi-synthetic macrolide and a unique microfilaricidal drug that is applied topically once monthly administered at 6-12 mg/ kg body weight. This dosage has an equivalent microfilaricidal activity as other macrolides, thus minimizing the transmission risk to new hosts [8]. It was hypothesized that treating microfilaria-positive dogs with macrolide agents would reduce the microfilaria load, thus reducing the disease transmission risk to new hosts. The present study was undertaken with the objective of comparing the efficacy of subcutaneously administered ivermectin with the topical application of selamectin.

## 2. Materials and methods

### 2.1. Animals and study location

The dogs presented for healthcare to the Veterinary Clinical Complex (VCC) of Veterinary College, Bhubaneswar, and government-run Veterinary Hospital at Sahid Nagar, Bhubaneswar, for the Animal Birth Control (ABC) Programme during an eight-months period from

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November 2014 to June next year, were recruited for the present study. The city, located at a latitude of 20.296059 and a longitude of 85.824539, has a tropical savanna climate. The temperature ranges from 11 to 44°C (52 to 111°F). It experiences four primary seasons: winter (December to February), when temperature drops to 11°C (52°F), summer (March to May), when temperatures can reach 44°C (111°F) or higher, monsoon from June to August, and post-monsoon from September to November. The annual average temperature is 27.4°C (81.3°F), and the monthly average temperature ranges from 22°C to 32°C (72 -90 °F). Summer months are hot and humid, with temperature, as low as 30°C. The maximum temperatures habitually exceed 104°F (40°C) during dry periods in May.

## 2.2. Screening for dirofilariasis

A total of 65 dogs were suspected of dirofilariasis, based on exhibiting clinical signs like dyspnoea, and a history of deep and prolonged coughing in the morning and evening hours, lethargy, and pale mucous membranes. Those included 43 males and 22 females, and there were 19, 28, 13, and 5 dogs in the age group of <3 years, 3 to <6 years, 6 to < 9 years, and 9 years or above (≥9 years), respectively (Table 1).The study included 12 Labradors, 10 Spitz, 21 indigenous breeds/ stray dogs, 6 Dobermans, 10 German Shepherds, and 6 Dachshunds. The dogs were examined for the presence of external parasites before the experiment. The dogs with a heavy external parasitic load were not recruited for the study. The planned study was conducted by professionally competent, VCI (Veterinary Council of India) -registered veterinary medicine practitioners. The percentage of infection in males, females, or age groups was calculated using the formula as given below.

$$\textbf{Infection } \% = \frac{\text{Number of microfilaria}-\text{positive dogs in a specific criterion (male/female/ age group)}}{\text{Total number of examined dogs in that respective variable}} \times 100\% \quad \textbf{(Equation 1)}$$

## 2.3. Experimental design

A total of fifteen dogs, five with blood samples negative for microfilaria (group I, Control) and 10 microfilaria-positive dogs, comprising five in group, were recruited for data analysis and reporting. The inclusion/exclusion of dogs was irrespective of age, sex, or vaccination status. The consent of the owner for the participation of his dog in the study was the only criteriaon. The data from 10 microfilaria-positive dogs, treated with either ivermectin (group II; n=5) or selamectin (group III; n=5), were finally used for further analysis and reporting. The dogs, whose owners gave consent to participate in the study, were presented for sampling as per the schedules, and volunteers who agreed to publish the data without disclosing personal details were only included for documentation. Some treated dogs were disqualified for their inclusion at a later date if the owner failed to comply with the schedules or there were issues in the collection of samples. The number of dogs in either of the treatment groups was rounded to only five in each for documentation. Group II dogs received injectable ivermectin (Hitek™ from Virbac Animal Health India, Borivali East, Mumbai, Maharashtra) at 50 µg/kg body weight subcutaneously on day 0, 7, and 14 by registered veterinary practitioners, and treated dogs were observed for any untoward side effects following injections [21]. Group III dogs were treated topically with the synthetic macrolide, selamectin (Revolution ™ from Pfizer Animal Health Division, New York) at 10 mg/kg body weight within the recommended dose range of 6 - 12mg/ kg, at weekly intervals for three doses. Ancillary treatments were administered in both treatment groups as and when required, and included bronchodilators, antibiotics, diuretics, and vitamin supplements.

## 2.4. Blood sampling

About 5 mL of blood sample was collected from each dog on days 0, 7, and 14 by venipuncture of the recurrent tarsal or cephalic vein. One milliliter of blood was transferred into a sterile vial containing anticoagulant, EDTA at a concentration of 1 mg/ 5 mL of blood [21]. The wet blood smear and thick blood smear examinations, along with the modified Knott's method, were employed for the screening of dirofilariasis. Sahli's acid hematin method was used for the estimation of hemoglobin. The total erythrocyte count (TEC) and total leukocyte count (TLC) were carried out by using a hemocytometer and Thomas fluid or Heyem's fluid, respectively as diluents. Giemsa's staining of a thin blood smear was done for the differential count, morphological study of blood cells, and identification of microfilaria. The packed cell volume (PCV) was assessed by Weintraub's hematocrit method. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were derived using hemoglobin, PCV, and TEC [21].

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## 2.5. Serum biochemical analysis

Another 4 mL of blood was transferred into test tubes without anticoagulant, and the tubes were allowed to stand in a slant position at room temperature for 3 to 4 hrs. The blood clot was carefully detached from the side wall of the test tubes by running a clean applicator stick around the inner surface of the tube with caution to avoid hemolysis. The test tubes were centrifuged at 1000 rpm for 2 minutes. The supernatant serum was pipetted out using an auto-pipette. and stored in autoclaved Eppendorf tubes in a deep freezer at -20°C. Serum AST, ALT, ALP, BUN, Creatinine, total protein, and blood glucose were analyzed using kits supplied by Crest Biosystem, Goa [22-26].

## 2.6. Microfilariae count

One milliliter of blood was mixed with 10 mL of 2% buffered formalin and centrifuged at 1000 rpm for 5 minutes for quantitative analysis of microfilariae [27]. One hundred microliters of sediment were mixed with equal parts of 1:1000 methylene blue stain. Twenty microliters of stained sediment were placed on a slide, layered with a coverslip, and examined under a microscope. The number of microfilariae viewed was multiplied by 10 and expressed as microfilariae /mL.

## 2.7. Statistical analysis

The data were analysed to express in mean ± S.E, and were statistically interpreted using SPSS-16 software package, employing one-way and repeated measure ANOVA, and post hoc analysis was done by Duncan multiple range test [28].

# 3. Results

## 3.1. Prevalence

Out of 65 suspected dogs screened for dirofilariasis, 42 dogs were diagnosed positive for blood microfilaria (Table 1). These 42 dogs included 15 non-descript / stray dogs, 10 Labradors, 8 Spitz, 6 German Shepherds, 2 Dobermans, and one Dachshund. The infection percent was comparatively higher in males (n=28/ 43; 65.1%) than in females (n=14/ 22; 63.6%). Among the 42 microfilaria positive dogs, the infection percentage was more in the age group of 3 to <6 years (22/ 28; 78.6%), followed by the dogs in the age group within 6 to < 9 years (8/13; 61.5%), below 3 years (11/ 19; 57.9%), then ≥9 years (1/ 5; 20.0%).

## 3.2. Therapeutic study

The negative status of the blood microfilaria in group I continued till the last observation made on day14, whereas the microfilaria load on day 0 (108.0 ± 15.94/ mL of blood) reduced significantly by day 7 (32.00±5.83/ mL of blood) in group II dogs, treated with ivermectin The microfilaria load further reduced to 2.00 ± 2.00 microfilaria/ mL of blood by day 14. Selamectin topical application reduced the microfilaria number to 74.0 ± 9.27/ mL of blood by day 7, and 66.81±8.12/ mL of blood by day 14 from day 0 counts of 88.00 ± 8.00 / mL (Figure 1). However, the decline in microfilaria count in group III was non-significant ($p \geq 0.05$). Thus, ivermectin injection at a weekly interval was effective in significantly reducing blood microfilaria load ($p < 0.05$), unlike topical application of selamectin.

## 3.3. Hematological changes

The hematological changes with respect to erythrocytic cell series in dogs on different observation days are shown in Table 2. The day 0 mean hemoglobin level in the control group was significantly higher than in either of groups, II and III. A significant rise in hemoglobin level was observed in group II over the period following treatment. However, the mean Hb level on day 14 (11.02±0.65 g/dL) remained statistically ($P < 0.05$) lower than that of group I. The total erythrocyte count (TEC) in the control group at three observation days was 6.82±0.24, 6.72±0.25, and 6.69±0.25 × 10^6/µL of blood, and these values were statistically comparable at $p \geq 0.05$.

**Table 1. The infection percentage with respect to sex and age groups following screening of dogs for blood microfilaria.**

| Number of | Total | Sex | | Age group | | | |
|---|---|---|---|---|---|---|---|
| | | Male | Female | <3yrs | 3 to <6 yrs | 6 to < 9 yrs | ≥9 yrs |
| Suspected dogs screened | 65 | 43 | 22 | 19 | 28 | 13 | 5 |
| Infected | 42 | 28 | 14 | 11 | 22 | 8 | 1 |
| Negative | 23 | 15 | 8 | 8 | 6 | 5 | 4 |
| Infection % | 64.6 | 65.1 | 63.6 | 57.9 | 78.6 | 61.5 | 20.0 |

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**Table 2. Hematological changes in dogs during treatment for dirofilariasis.**

| Parameters | Group (n=5) | Days after the start of the treatment | | |
|---|---|---|---|---|
| | | 0 | 7 | 14 |
| Hb (g/dL) | I | 14.24±0.44 $^{aB}$ (12.89-15.50) | 14.92±0.37 $^{aB}$ (13.50-15.56) | 14.46±0.39 $^{aB}$ (13.00-15.20) |
| | II | 8.94±0.85 $^{aA}$ (5.89-10.50) | 10.13±0.76 $^{abA}$ (7.50-11.67) | 11.02±0.65$^{bA}$ (8.90-12.50) |
| | III | 9.09±0.64$^{aA}$ (7.50-11.25) | 9.38±0.63$^{aA}$ (7.89-11.50) | 10.04±0.58$^{aA}$ (8.50-12.00) |
| TEC ($10^6$/µL) | I | 6.82±0.24 $^{aB}$ (6.20-7.50) | 6.72±0.25 $^{aB}$ (6.20-7.58) | 6.69±0.25 $^{aB}$ (6.12-7.34) |
| | II | 4.00±0.36$^{aA}$ (3.00-4.85) | 4.76±0.19$^{aA}$ (4.20-5.20) | 5.46±0.15$^{aA}$ (4.90-5.80) |
| | III | 4.26±0.27$^{aA}$ (3.80-5.30) | 4.50±0.27$^{aA}$ (3.90-5.50) | 4.90±0.31$^{bA}$ (4.20-6.00) |
| PCV (%) | I | 41.00±1.18$^{aA}$ (37.00-44.00) | 43.55±0.98$^{aB}$ (40.50-45.60) | 41.88±0.78$^{aB}$ (39.45-44.35) |
| | II | 30.26±1.65$^{aA}$ (25.00-35.00) | 32.90±1.03$^{abA}$ (30.00-35.00) | 36.50±0.74$^{bA}$ (34.50-38.00) |
| | III | 30.30±2.08$^{aA}$ (26.50-38.00) | 31.70±1.69$^{aA}$ (28.00-38.00) | 34.40±1.03$^{aA}$ (32.00-38.00) |
| MCV (fL) | I | 60.17±1.16$^{aA}$ (58.01-64.62) | 64.98±1.74$^{aA}$ (60.16-70.98) | 62.78±1.48$^{aA}$ (58.17-65.46) |
| | II | 77.24±5.60$^{bB}$ (62.50-92.11) | 69.42±2.79$^{abA}$ (62.50-77.78) | 67.00±1.78$^{aA}$ (61.61-71.43) |
| | III | 71.20±2.22$^{aB}$ (63.10-76.23) | 70.76±2.81$^{aA}$ (63.64-80.77) | 70.75±2.28$^{aA}$ (63.33-76.19) |
| MCH (pg) | I | 20.88±0.19 (20.44-21.54) | 22.28± 0.80 (20.05-24.54) | 21.67±0.67 (20.08-23.82) |
| | II | 22.14±1.55 (19.27-27.63) | 21.23±1.20 (17.86-24.89) | 20.18±1.02 (16.48-22.32) |
| | III | 21.36±0.78 (19.62-23.58) | 20.82±0.50 (19.32-22.22) | 29.09±0.83 (19.40-21.88) |
| MCHC (%) | I | 34.73±0.36$^{aB}$ (33.33-35.24) | 34.27±0.61$^{aB}$ (33.33-36.52) | 34.51±0.55$^{aB}$ (32.95-36.39) |
| | II | 29.28±1.55$^{aA}$ (23.56-32.81) | 30.68±1.65$^{aA}$ (24.19-33.34) | 30.24±1.81$^{aA}$ (23.42-33.78) |
| | III | 30.08±1.21$^{aA}$ (25.86-33.19) | 29.59±1.20$^{aA}$ (25.05-32.36) | 29.10±0.83$^{aA}$ (26.56-31.58) |

The values are given as Mean ± S.E. The values in parentheses indicate a range. Values with dissimilar superscripts, small letters in row and capital letters in column, vary at $p<0.05$. Hb (g/dL) –Hemoglobin (gram/ deciliter); TEC – total erythrocyte counts; PCV – packed cell volume; MCV – Mean Corpuscular Volume; MCH – Mean Corpuscular Hemoglobin; MCHC– Mean corpuscular hemoglobin concentration.

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The mean total erythrocyte counts (TEC) of group II and III on day 0 were statistically ($p<0.05$) lower than those of group I. The TEC improved in group II and III at subsequent observations following treatment. However, the mean level noted for groups II and III on day 0, 7, and 14 remained statistically comparable ($p\geq0.05$). Group II and III dogs had a mean PCV level on day 0 that was significantly lower than that of group I. A significant improvement in PCV level was recorded in group II dogs on day 14 (36.50%±0.07) as compared to the day 0 level (30.26%±1.65). However, dogs treated with selamectin via dermal application (group III) continued to have PCV levels statistically comparable at different observation periods.

The erythrocytic indices namely, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), are shown in Table 2. The mean MCV level in dirofilaria-positive dogs was significantly higher than the mean level of the control group on day 0. The mean level in groups II and III declined significantly at subsequent observation periods. The mean corpuscular hemoglobin level in control and dirofilaria-positive dogs at different periods of observation remained statistically comparable ($p\geq0.05$) and ranged between 16.48 pg and 22.32 pg. The mean corpuscular hemoglobin concentration (%) in control animals of group I on day 0, 7, and 14 was statistically comparable. The affected dogs had a mean MCHC value of 29.28%±1.55, and 30.08%±1.21in groups II and III, respectively. The mean level was statistically ($p<.05$) lower than that of the unaffected dogs, irrespective of the treatment. The mean level at subsequent observation periods remained comparable to the respective day 0 value.

The mean total leukocyte counts in groups II and III before treatment were significantly ($p<0.05$) higher than those in the control group (10.66±1.05 × 10^3/µL ). However, the mean TLC declined significantly ($p<0.05$) in group II dogs at subsequent observations on days 7 and 14 (Table 3). The neutrophil counts in groups II and III were significantly higher ($P<0.05$) than those of the control dogs. The treatment with ivermectin in group II dogs reduced the percentage of neutrophils at subsequent observations on days 7 and 14. However, the reduction in neutrophil percentage in group III dogs was statistically comparable at P<0.05%. Lymphocyte % in microfilaria positive dogs (9.40±2.09 % and 13.20±1.69% in group II and III, respectively) were statistically ($p<0.05$) lower than those of the respective values of the negative control group. There was a significant improvement in the percentage of lymphocytes in ivermectin- or selamectin-treated dogs. However, the lymphocyte % on day 14 in group III dogs was significantly ($P<0.05$) lower than that of groups I and II. The eosinophil % was higher in affected dogs compared to controls (Table 3).

### 3.4. Biochemical changes

Table 4 shows serum biochemical changes before and following treatment of microfilaria-positive dogs. The mean glucose levels in microfilarial positive dogs (groups II and III) were significantly ($P<0.05$) lower than the mean level in blood microfilaria negative dogs. The subcutaneous administration of ivermectin increased the blood glucose level in group II dogs, and the mean level recorded on day 14 for the group was significantly higher than the day 0 observation, unlike in group III, where the mean levels recorded at three periods of observation were statistically comparable ($p\geq0.05$). The blood urea nitrogen (mg/dL) and creatinine (mg/dL) levels as well as the activity of serum enzymes such as AST, ALT, and ALP (IU/L) in affected dogs, remained significantly higher than those of control dogs. The serum total protein concentration was non-significantly elevated in dogs with dirifilariasis. The mean total protein level values recorded at different observation periods in three different groups remained statistically comparable ($p\geq0.05$) . However, the minimum and maximum values recorded for dogs with microfilarial load, irrespective of the treatment at different observation periods, were numerically higher than the respective values in control dogs. The treatment with ivermectin through the subcutaneous route significantly ($p<0.05$) reduced BUN in group II, and non-significantly in group III dogs treated with selamectin, which continued to remain significantly higher than the control group at the respective observation period.

## 4. Discussion

The majority of the infected dogs suffer from subclinical dirofilariasis. The present investigation examined the blood samples from the suspected dogs for the presence of microfilaria. A combination of both antigen testing and microscopic detection of microfilaria in peripheral blood has been recommended for the diagnosis of heartworm disease. The antigen test may even detect adult *Dirofilaria immitis* in the pulmonary artery in microfilaria-

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negative dogs, more specifically so in occult or mono-sex parasitism [1]. However, the detection of adult worms in the heart or subcutaneous tissues either during a surgery or after death is considered irrefutable [29, 30]. Besides, microfilariae induce considerably less pathological effects on the host than adult parasites, as pulmonary artery thromboembolism is caused by the dead worms. The US Food and Drug Administration (FDA) has approved the regular use of preventive drugs throughout the year to prevent heartworm infection by reducing the transmission risk [1].

The distribution of dirofilariasis and its prevalence is changing due to climate change, with cases increasing in geographical areas where the status was negative beforehand or with low prevalence [29, 31]. As many as 70 mosquito species act as vectors, thus stressing the role of climate factors in the transmission of the disease. So, climatic and ecological elements influence the transmission. It is generally anticipated that climate change will impact the spread of vector-borne diseases in Europe, since arthropod vectors are particularly sensitive to climatic factors.

**Table 3. Total Leukocyte count (TLC) and Differential counts (DC) in dogs during treatment for dirofilariasis.**

| Parameters | Group (n=5) | Days after the start of the treatment | | |
|---|---|---|---|---|
| | | 0 | 7 | 14 |
| TLC ($10^3$/µL) | I | 6.82±0.24 $^{aB}$ (6.20-7.50) | 6.72±0.25 $^{aB}$ (6.20-7.58) | 6.69±0.25 $^{aB}$ (6.12-7.34) |
| | II | 4.00±0.36$^{aA}$ (3.00-4.85) | 4.76±0.19$^{aA}$ (4.20-5.20) | 5.46±0.15$^{aA}$ (4.90-5.80) |
| | III | 4.26±0.27$^{aA}$ (3.80-5.30) | 4.50±0.27$^{aA}$ (3.90-5.50) | 4.90±0.31$^{bA}$ (4.20-6.00) |
| Neutrophils (%) | I | 63.80±1.66$^{aA}$ (59.00-68.00) | 63.80±1.93$^{aA}$ (58.00-70.00) | 65.20±1.66$^{aA}$ (60.00-70.00) |
| | II | 87.40±2.36$^{cB}$ (82.00-96.00) | 79.40±1.54$^{bB}$ (76.00-85.00) | 73.40±1.36$^{aB}$ (69.00-77.00) |
| | III | 83.40±2.73$^{aB}$ (77.0 – 92.0) | 82.40±2.84$^{aB}$ (75.00-90.00) | 80.00±1.95$^{aC}$ (75.00-85.00) |
| Lymphocytes (%) | I | 35.40±1.86$^{aB}$ (30.00-40.00) | 37.20±1.28$^{aB}$ (34.00-41.00) | 33.60±1.66$^{aC}$ (29.00-39.00) |
| | II | 9.40±2.09$^{aA}$ (3.00 -14.00) | 18.60±1.29$^{bA}$ (14.00-21.00) | 25.60±1.47$^{cB}$ (21.00-30.00) |
| | III | 13.20±1.69$^{aA}$ (7.00-17.00) | 14.40±1.96$^{abA}$ (8.00-19.00) | 17.40±1.69$^{bA}$ (15.00-24) |
| Eosinophils (%) | I | 0.40±0.24 (0.00-1.00) | 0.40±0.24 (0.00-1.00) | 0.80±0.20 (0.00-1.00) |
| | II | 3.00±1.00 (3.00-4.00) | 1.80±0.58 (0.00-3.00) | 0.80±0.20 (0.00-1.00) |
| | III | 2.80±1.24 (0.00-7.00) | 2.6±1.21 (0.00-7.00) | 1.64±0.29 (0.00-6.00) |
| Monocytes (%) | I | 0.40±0.24 (0.00-1.00) | 0.60±0.24 (0.00-1.00) | 0.40±0.24 (0.00-1.00) |
| | II | 0.20±0.20 (0.00-1.00) | 0.20±0.20 (0.00-1.00) | 0.20±0.20 (0.00-1.00) |
| | III | 0.60±0.40 (0.00-2.00) | 0.40±0.40 (0.00-2.00) | 0.40±0.40 (0.00-2.00) |

The values are given as Mean ± S.E. The values in parentheses indicate a range. Values with dissimilar superscripts, small letter in row and capital letter in column, vary at $p<0.05$. TLC – total leukocyte counts.

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**Table 4. Biochemical changes and serum enzymatic activities in dogs during treatment for dirofilariasis.**

| Parameters | Group (n=5) | Days after the start of the treatment | | |
|---|---|---|---|---|
| | | 0 | 7 | 14 |
| Glucose (g/dL) | I | 84.39±4.99$^{aB}$ (69.56-95.89) | 83.14±4.59$^{aB}$ (70.34-94.00) | 83.84±4.21$^{aB}$ (70.45-92.45) |
| | II | 48.22±4.04$^{aA}$ (38.71-60.12) | 57.10±5.54$^{abA}$ (27.13-39.18) | 67.59±4.41$^{bA}$ (58.45-81.35) |
| | III | 54.20±4.44$^{aA}$ (40.56-66.28) | 54.95±3.60$^{aA}$ (29.78-64.89) | 55.10±2.54$^{aA}$ (48.23-60.47) |
| BUN (mg/dL) | I | 23.84±2.77$^{aA}$ (15.85-30.67) | 23.70±2.12$^{aA}$ (17.23-28.45) | 23.01±2.33$^{aA}$ (17.34-29.14) |
| | II | 44.25±2.87$^{bB}$ ( 39.45-55.12) | 34.42±1.96$^{abB}$ (27.13-39.18) | 29.94± 0.67$^{aB}$ (27.55-31.23) |
| | III | 40.34±4.63$^{bA}$ (30.44-55.98) | 39.16±4.35$^{aB}$ (29.78-53.87) | 38.99±4.65$^{aB}$ (29.00-54.20) |
| Creatinine (mg/dL) | I | 1.10±0.05$^{bA}$ (0.95-1.23) | 1.19±0.11$^{Abc}$ (1.01-1.45) | 1.04±0.02$^{aA}$ (0.98-1.11) |
| | II | 2.03±0.17$^{bB}$ (1.56-2.58) | 1.76±0.08$^{abA}$ (1.54-1.95) | 1.37±0.09$^{aAB}$ (1.14-1.67) |
| | III | 1.83±0.31$^{aB}$ (0.96-2.87) | 1.79±0.33$^{aA}$ (0.80-2.87) | 1.70±0.27$^{aB}$ (0.82-2.45) |
| Total Protein (g/dL) | I | 6.49±0.60 (4.56-8.08) | 6.49±0.55 (4.70-8.00) | 6.45±0.50 (4.87-7.89) |
| | II | 8.33±0.75 (6.58-10.89) | 7.81±0.65 (6.12-9.98) | 7.32±0.38 (6.40-8.56) |
| | III | 8.60±0.77 (6.14-10.67) | 8.29±0.96 (6.11- 11.00) | 8.17±0.85 (6.00-10.45) |
| AST (IU/L) | I | 14.02±1.26$^{aA}$ (10.82-18.45) | 14.07±1.18$^{aA}$ (11.34-17.46) | 14.34±0.83$^{aA}$ (12.53-16.98) |
| | II | 40.53±7.15$^{bB}$ (27.41-66.98) | 32.14±3.68$^{bB}$ (24.56-44.56) | 22.35±1.84$^{aA}$ (18.23-28.64) |
| | III | 39.88±1.46$^{aB}$ (35.25-43.78) | 39.15±0.98$^{aB}$ (36.78-41.67) | 37.05±1.13$^{aB}$ (34.24-41.44) |
| ALT (IU/L) | I | 17.82±1.56$^{aA}$ (11.67-20.19) | 18.00±1.08$^{aA}$ (13.78-19.56) | 17.52±1.36$^{aA}$ (12.34-20.45) |
| | II | 46.67±9.21$^{bB}$ (27.63-78.45) | 36.84±5.62$^{bB}$ ( 24.67-55.09) | 20.24±2.17$^{aA}$ (14.75-27.44) |
| | III | 41.90±4.06$^{aB}$ (29.75-52.51) | 40.98±3.81$^{aB}$ (30.45-50.78) | 39.60±3.53$^{aB}$ (29.67-47.78) |
| ALP (IU/L) | I | 14.93±1.12$^{aA}$ (11.67-18.27) | 15.43±1.12$^{aA}$ (12.45-19.34) | 15.38±0.71$^{aA}$ (13.67-17.76) |
| | II | 61.16±7.66$^{bB}$ ( 47.34-88.25) | 48.88±5.84$^{bB}$ ( 37.45-69.45) | 31.75±2.79$^{aB}$ (22.71-38.45) |
| | III | 55.78±6.77$^{aB}$ (38.16-75.99) | 55.05±6.37$^{aB}$ (39.45-74.27) | 53.23±6.74$^{aC}$ (35.78-73.02) |

The values are given as Mean ± S.E. The values in parentheses indicate a range. Values with dissimilar superscripts (small letter in row and capital letter in column), vary at p≤0.05. BUN – Blood Urea Nitrogen; AST - Aspartate Aminotransferase; ALT- Alanine Aminotransferase; ALP – Alkaline Phosphatase.

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Weather influences the development and sustenance of arthropod vectors, but climate change is one of several factors that impact on vector habitat and growth. Therefore, climate change effects have been incriminated in the spread of vector-borne diseases [32].

The present study confirmed the presence of microfilaria in the blood samples from 42 suspected dogs using the modified Knott's method and wet blood film examination. The earlier published report from our laboratory on Polymerase Chain Reaction (PCR) using these 65 blood samples, followed by gel electrophoresis of the PCR products of 42 microfilaria-positive samples, documented 15 single bands and 26 double bands, and one with a single lower band. The upper band of the double bands and the single band (n=26+15=41) were positive for *D. immitis*, as confirmed by sequence analysis of the purified PCR products [33].

The male dogs were at a higher risk, nearly twice that of the females as reported earlier [34-36]. However, there are reports that disprove the role of sex, age, size, breed, body weight, and hair coat on the prevalence of dirofilariasis [37]. Among the 42 microfilaria-positive dogs, the maximum cases were observed in the age group between 3 to <6 years. The risk of infection increased in the age group of 6 to <9 years, but declined in dogs that were 9 years and above [37]. There is no explicit explanation for why this age group and male dogs had a higher prevalence of microfilaria and are at a higher risk. This may be attributed to more mosquito bites and an increased risk of harbouring the parasite due to the movement of dogs and the increased outdoor activity [38].

The present investigation, which reveals anaemia, a decreased total erythrocytic count, and an increased leucocytic count, confirms the earlier findings [37]. There was 39.40% decline in total erythrocytic count and 36.70% decline in hemoglobin level, suggesting that the hemoglobin content of the erythrocyte in the circulating blood declines in dirofilariasis. Dirofilariasis causes anaemia through intravascular hemolysis as a large number of adult heartworms and microfilaria obstruct the blood flow and disturb the erythrocytes, resulting in hemolytic and regenerative anaemia, hemoglobinemia, and hemoglobinuria [9, 39, 40]. However, the decrease in hemoglobin concentration as compared to total erythrocytic count was associated with non-significant changes in MCH level. Regenerative anaemia in dirofilariasis was also evident from a significant increase in the mean value of MCV accompanied by macrocytic hypochromic anaemia with reduction in erythrocyte count, hemoglobin concentration, and PCV. The macrocytosis and hemochromasia have been attributed to reticulocytosis observed in microfilaria-infested dogs [41]. Therefore, the present findings are attributed to the hemolysis of erythrocytes hemoglobinemia resulting from the destructive motility of microfilaria.

Leukocytosis in dirofilariasis was associated with neutrophilia, and lymphopenia, with non-significant changes in eosinophil, monocyte, and basophil counts. The higher blood neutrophil counts are attributed to increased phagocytic activities for the removal of tissue breakdown products of microfilaria. Similarly, neutrophilic leukocytosis was observed in dogs with dirofilariasis [41]. A non-significant increase in eosinophilic count may be due to the increased sensitivity of the host to the proteins of parasites as an immune phenomenon [42]. The intense antigenic stimulation increases the demand for leucocytes in the circulation leading to the transformation of lymphocytes into plasma cells for antibody production, resulting in hematological findings of lymphocytosis. However, the present study shows lymphopenia was a clear hematological change in dogs with circulating microfilaria.

Biochemical investigations revealed hypoglycemia, elevated BUN, creatinine, and a non-significant increase in total protein. Hypoglycemia has been reported earlier in *Dipetalonema reconditun* infection [40], and such findings in the present study may be attributed to glucose consumption by the parasite and hepatic insufficiencies due to circulatory disturbances. The hypoglycemia originating from hepatic insufficiency was further substantiated by increased activities of liver-associated serum enzymes such as AST, ALT, and ALP. The damage to the hepatocytes results in the leakage of these enzymes into the blood vascular system, thereby increasing the activities of these enzymes [9, 43, 44]. Thus, the present finding confirms hepatic damage contributing to inappetence in affected dogs with poor body condition. The increase in BUN level further substantiated hepatic damage. Blood urea nitrogen (BUN) and creatinine are the markers of renal function. The increase in BUN and creatinine in the present experiment suggested renal dysfunction, which could be attributed to circulatory disturbances caused by circulating

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microfilaria and immune complex formation and their deposition in the renal glomerular capsules, thereby reducing the glomerular filtration rate (GFR). Increased ALP activities and BUN levels have also been previously reported in heartworm disease [5]. That was associated with severe renal dysfunction, metabolic acidosis, and intravascular hemolysis [39, 45]. A non-significant increase in serum protein level and hypoproteinemia could be attributed to an increase in gamma globulin concentration as an immunological response to parasitic antigen or release of hemoglobin from the destroyed erythrocytes, which was further validated by increased MCV and decreased Hb level [40, 46].

The number of heartworm cases in dogs are the rise by over 20% from 2013 to 2016 [47]. A survey of more than 18, 000 veterinary clinics in the USA diagnosed more than 240,000 dogs and 3000 cats infected with *D. immitis* [8]. Such an alarmingly high level of incidence established the compliance failure [5-7]. Dirofilariasis has a steady increasing trend in human beings in European and American countries [5]. Several human cases of dirofilariasis have been reported in different parts of Iran over than the past decades, suggesting zoonotic significance of the disease, and necessitating strict control measures of the disease in animal hosts [48, 49].

Despite many advances in the development of preventive methods, dirofilariasis still causes large mortality in affected animals [8]. Clearing adult heartworm requires intramuscular administration of three injections of melarsomine, an organic arsenic compound. Chronic use of certain macrocyclic lactones along with doxycycline, is effective, and it is used when melarsomine is not available or cannot be used [33]. Despite the commercial availability of several drugs that kill immature heartworms through veterinary prescriptions in the United States, heartworm disease continued as a common problem [42]. The recent control strategies against dirofilariasis in dogs are based on the intermittent use of microfilaricidal drugs aimed to the break at breaking the life cycle of the parasite and reducing the incidence of clinical disease [50]. Doxycycline has been reported to possess anti-microfilaria effects [27, 51]. However, the advent of macrolide agents, namely, ivermectin, milbemycin oxime, moxidectin, and selamectin has contributed to the control of parasitic infections that interrupt larval development during the first two months of infection without any adverse reactions, and those are considered superior to diethyl carbamizene citrate (DEC) [52]. Ivermectin is a chemical derivative of avermectin B1, which is produced from *Streptomyces* species. It is effective against a broad range of endo- and ecto-parasites, with outcomes comparable following administration through oral or subcutaneous route [53]. It is marketed as a heartworm preventive to be used once month. Despite the gradual destruction of microfilaria, mild adverse reactions such as transient diarrhea upon its administration to dogs with microfilaremia, it is still superior to the adulticidal use of thiacetarsamide, which is associated with potential arsenical toxicity and local inflammatory reactions, exacerbating inflammatory reactions and formation of massive thromboembolisms [28]. Selamectin, a semi-synthetic macrolide, is unique for its spectrum of anti-microfilaria activity parallel to other macrolides [8]. A wide margin of safety has been demonstrated in young and adult dogs following its use [54]. The efficacy of selamectin in declining microfilaria load offered advantages in minimizing the adverse reactions due to dead or dying microfilaria in large numbers.

The subcutaneous administration of ivermectin reduced the microfilaria load in the circulating blood as reported earlier [43]. Selamectin also reduced the number of blood microfilariae in circulation, but the decline was not significantly different from the day 0 level, clearly suggesting that ivermectin subcutaneous administration was more efficacious than topical application of selamectin. These agents are superior to diethylcarbamazine (DEC) in terms of convenience and adverse reactions when administered in dogs with circulating microfilaria. The findings of the present study, regarding hematobiochemical alterations and serum enzymatic activities, further confirm the restoration of health with the reduction in blood microfilaria counts. The hemoglobin, TLC, and PCV levels showed a gradual increase and were almost normal by day 14 post-treatment. This is attributed to a reduction in non-premature destruction of erythrocytes. Similarly, there was an improvement in erythrocytic indices and serum biochemical parameters, particularly BUN and creatinine levels, as well as activities of serum enzymes related to hepatic function. Further studies involving a larger number of microfilaria-positive dogs for a longer duration are required to assess the efficacy of ivermectin and selamectin in restoring the hematological and clinicobiochemical changes.

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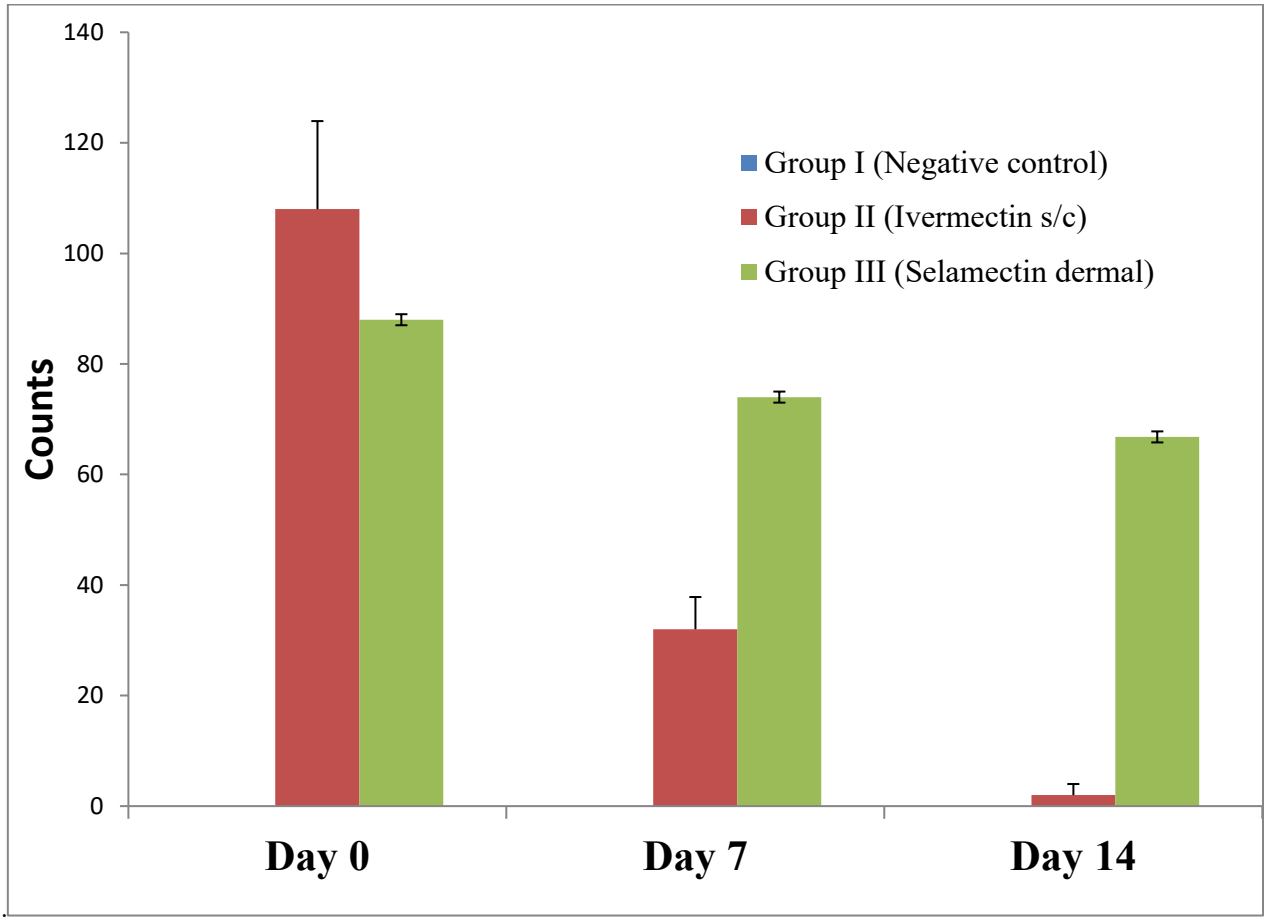


**Figure 1.** Microfilaria counts/mL of blood following treatments with ivermectin or selamectin in dogs.

## 5. Conclusions

It was concluded that subcutaneous administration of two doses of ivermectin at 50 µg/kg body weight at weekly interval had better efficacy than dermal application of selamectin in restoring the clinicopathological parameters towards normalcy, and reducing the blood microfilaria counts, thus reducing the spread of the disease, and such preventive measures can be employed to reduce the transmission risk in heartworm endemic areas.

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## Conflicts of interest

There were no conflicts of interest.

## Ethical approval

This clinical study was carried out by registered veterinary physicians, observing CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines, for the welfare and good health of the animals. No invasive or painful procedures were performed. The owner's consent was taken prior to the sampling of the dog for conduct of the tests.

## Author contribution

1. Manisha Das, drmanishadas28@gmail.com: Executed the work.

2. *Ramesh Chandra Patra, rcpatra@gmail.com: Planned the experiment and supervised the work.

3. Rajasri Sahoo, rajasrisahoo@gmail.com: Wrote the manuscript.

4. Mirashree Pati, pati.mirashree@gmail.com: Edited the manuscript.

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## Author information

**Corresponding Author:** R. C Patra*

**E-mail:** rcpatra@ouat.ac.in / rcpatra@gmail.com

**ORCID iD:** 0000-0002-0418-7509

## Data availability

Data will be available on request.

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