CLINICO-THERAPEUTIC STUDIES ON CANINE MICROFILARIOSIS

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Thesis submitted in partial fulfillment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled "**CLINICO-THERAPEUTIC STUDIES ON CANINE MICROFILARIOSIS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associate ship, fellowship or other similar title, of any other University or Society.

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Dedicated to my
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Introduction

1. INTRODUCTION

Dog, a companion of mankind is prone to many diseases due to import, mixing of animals and management errors, among which parasitic diseases constitute a major problem. Disease due to filarial nematodes is common throughout many parts of the world where the climate permits an abundant and susceptible population of intermediate insect host. The two most important canine filariae are *Dirofilaria repens* and *Dirofilaria immitis*. Pathological role of *Dirofilaria immitis* has been documented extensively, but lack of clinical manifestations and recognized pathogenicity of *Dirofilaria repens* infection in dogs has lead to only brief mention in most veterinary texts as a harmless worm residing in the subcutaneous connective tissue (Soulsby, 2005). The prevalence of canine filariasis in the state is also believed to be high. Thus it increases its zoonotic potential. The increased prevalence of filarial infection in dogs and the low level of awareness regarding this infection among local residents together with increasing popularity of acquiring dogs as pets in this country highlights the need for importance of public awareness of this infection especially among dog owners. Such measures will lead to early detection and proper management of this condition in dogs which inturn could lead to reduction of reservoir of infection for human filariasis. At this juncture, it would be advisable to know the status of human lymphatic filariasis in the state also. In recent years the incidence of human lymphatic filariasis is also found to be high inspite of the Mass Annual Single dose Diethyl Carbamazine Administration (MDA) programme launched by WHO.

Human lymphatic filariasis (LF) is caused by infection with nematode parasite *Wuchereria bancrofti* and *Brugia malayi*. Though not fatal the disease is responsible for considerable sufferings, deformity and disability. The WHO has launched a global programme for the elimination of lymphatic filariasis (GPELF) by the year 2020 by Mass Annual Single dose Diethyl Carbamazine (MDA) administration. The performance of first round MDA programme launched in

Kerala during the year 2005 was not satisfactory and main reasons for poor performance were inadequate public awareness, fear of drug reactions etc. (Showketh Ali, 2001). However one pivotal element for the success of any eradication/control programme is the control of infection in reservoir host if any. Malayan filariasis caused by *Brugia malayi* is endemic in South of Thailand where domestic cats serve as the major reservoir host. It has been postulated that filariasis control programme in this area is adversely affected by the involvement of reservoir host. This scenario may also be responsible for the poor performance of MDA programme in Kerala. High prevalence of filariasis in human beings together with increasing incidence of microfilariosis in dogs with clinical signs similar to that of human lymphatic filariasis and poor performance of MDA programme prompted to investigate the natural progression of filarial infection in dogs and its zoonotic importance. Hence the present study was carried out with the following objectives.

i) To study the epidemiology and clinical signs of canine microfilariosis

ii) To compare the microfilaricidal efficacy of oral ivermectin and levamisole hydrochloride therapy.

Review of Literature

2. REVIEW OF LITERATURE

The filarid worms belongs to super kingdom *Eukaryota*, kingdom *Metazoa*, phylum *Nematoda*, class *Chromadorea*, order *Spirurida*, super family *Filarioidea* and family *Onchocercidae*. The family *Onchocercidae* consists of several genera. Genera of great parasitic importance includes *Dirofilaria*, *Dipetalonema*, *Brugia*, *Wuchereria* and *Loa*. The genus *Dirofilaria* consists of *Dirofilaria repens* and *Dirofilaria immitis*. The genus *Dipetalonema* consists of *Dipetalonema reconditum, Dipetalonema dracunculoides, Dipetalonema gracile* etc. Important species belonging to *Brugia* genus are *Brugia malayi, Brugia pahangi, Brugia patei* and *Brugia timori. Loa loa* belongs to genus *Loa* and *Wuchereria bancrofti* belongs to genus *Wuchereria* (Soulsby, 2005).

2.1. GEOGRAPHICAL DISTRIBUTION OF FILARIAL NEMATODES

2.1.1. World Scenario

Kelly (1979) opined that *Brugia pahangi* was a natural parasite of dogs and cats of Africa and Far East.

Yoshimura and Akao (1985), Boreham (1988) and Sukpanichnant *et al.* (1998) reported human pulmonary dirofilariasis caused by *Dirofilaria immitis* in Brazil, Italy, France, Greece, Spain, Ukraine, Russia, The united states, Australia, Japan and Thailand.

WHO (1992) reported that human filariasis caused by *Brugia malayi* is still a public health problem in many countries of Asia especially India, Indonesia, Malaysia, Philippines, Srilanka and Thailand.

Blagburn (1994) observed three species of microfilariae producing canine microfilariosis in United States namely *Dirofilaria immitis, Dipetalonema reconditum* and *Dirofilaria striata.*

Ottesen and Ramachandran (1995) opined that the filarial parasites *Wuchereria bancrofti* and *Brugia malayi* were the major cause of human lymphatic filarasis in the tropical and subtropical regions of the world with an estimated 120 million people infected and an additional 900 million people at risk of infection.

Pampiglione *et al.* (1995) stated that subcutaneous dirofilariasis due to *Dirofilaria repens* was a helminthic zoonosis, widely distributed throughout Europe, Asia and Africa.

Michael and Bundy (1997) opined that although 90% of the lymphatic filariasis cases reported world wide were caused by *Wuchereria bancrofti*, a further 10% were attributed to brugian filariasis, which were restricted to the Asian region.

Division of filariasis (1998) reported mainly two physiological types of *Brugia malayi ie* the nocturnally subperiodic and diurnally subperiodic types in human beings in Southern Thailand.

Muro *et al.* (1999) opined that canine dirofilariasis was endemic in northern Italy and other Eurasian foci involving both *Dirofilaria immitis* and *Dirofilaria repens*.

Lymphatic filariasis (LF) was considered as a leading cause of permanent and long term disability and the World Health Organization has launched a global programme to eliminate lymphatic filariasis as a public health problem by the year 2020 (Ottesen , 2000).

Kanjanopas *et al.* (2001) identified *Brugia malayi* in a naturally infected cat from Narathiwat province, Southern Thailand by morphometry and acid phosphatase staining characteristics of microfilaria and adult morphology.

Fischer *et al.* (2002) stated that *Brugia timori* was limited to some Indonesian islands within Lesser Sunda archipelago and the global prevalence of

this species appeared very small in comparison with those of *Wuchereria bancrofti* and *Brugia malayi.*

Mallick and Ittyerah (2003) reported *Dirofilaria repens* as the sole cause of subcutaneous dirofilariasis in humans in Asia including India.

Lek-Uthai *et al.* (2004) revealed that the type of *Brugia malayi* expression in humans and cats in Thailand was similar and nocturnally subperiodic.

Byeon *et al.* (2007) observed the high prevalence of *Dirofilaria immitis* infection in pet dogs of Korea using antigen detection tests.

Lee *et al.* (2007) stated that *Dirofilaria repens*, found in subcutaneous tissue was a common parasite of dogs and cats in Europe, Africa and Asia. *Dirofilaria immitis*, the dog heart worm was distributed world wide in tropical, subtropical and temperate zones.

Tarello (2008) reported that dogs were natural hosts of *Dirofilaria repens* and acted as animal reservoirs for the zoonotic filariasis in human beings in Kuwait .

2.1.2. India

Chatterjee (1980) reported that *Wuchereria bancrofti* and *Brugia malayi* were the major filarial parasites of human beings in India.

Tewari *et al.* (1995) stated that diurnally subperiodic *Wuchereria bancrofti* was prevalent in Nicobar group of Islands.

ICMR (2002) conducted surveys regarding the prevalence of lymphatic filariasis in India and found that 22 out of 25 states were endemic for filariasis and the states of Bihar, Andra Pradesh, Orissa, Tamilnadu, West Bengal, Maharastra, Uttar Pradesh, Kerala and Gujarat contributed to about 95% of the total burden of filariasis.

Ananda and D'souza (2007) reported *Dirofilaria repens* and *Dipetalonema reconditum* from dogs in Karnataka.

2.1.3. Kerala

Joseph *et al.* (1967) studied the epidemiological aspects of lymphatic filariasis in the coastal belts of Kerala and reported that Kollam, Allepey, Ernakulam and Thrissur districts were highly endemic for this disease.

Valsala and Bhaskaran (1974) studied dirofilariasis in dogs and identified *Dirofilaria imitis* in dogs in Calicut district.

Saseendranath *et al.* (1986) reported that the incidence of canine microfilariosis in Thrissur and the microfilariae were morphologically identified as *Dirofilaria immitis*.

Radhika (1997) identified microfilaria of *Dirofilaria repens* as the causative agents of canine microfilariosis in Thrissur district.

Rajendran *et al.* (1997) stated that *Wuchereria bancrofti* and *Brugia malayi* were prevalent in human beings in Thrissur district.

Suprabha and Devada (2003) opined that microfilariae of *Dirofilaria repens* was the only larval form encountered in dogs in and around Trivandrum district.

Sabu *et al.* (2005) conducted a study on dirofilariasis in dogs and humans and identified *Dirofilaria repens* as the sole cause of canine microfilariosis in Kerala.

2.2. EPIDEMIOLOGY

2.2.1. Age

Jackson (1969) opined that venacavae or liver failure syndrome due to dirofiariosis occurs most frequently in dogs of three to five years old.

Martin and Collins (1985) surveyed that prevalence of *Dirofilaria immitis* and *Dipetalonema reconditum* microfilariae in grey hounds of Australia and opined that *Dirofilaria immitis* was most frequently found in dogs aged between 1.5 to 2 years and *Dipetalonema reconditum* in dogs up to12 months of age.

Radhika (1997) observed a higher prevalence of *Dirofilaria repens* in dogs of 6 months to 6 years than aged dogs while Suprabha and Devada (2003) noticed a higher prevalence of infection in dogs above six years of age than the younger ones.

Ananda and D'souza (2007) reported higher infection rates in dogs between two to seven years of age.

Miterpakova *et al*. (2008) stated that canine dirofilariosis was more often detected in animals aged over 3 years when compared with younger dogs.

2.2.2. Sex

Wallenstein and Tibola (1960) reported that male dogs were more prone to infection than the females.

Natural infections of *Dipetalonema reconditum* occur in males about twice as frequently as in females (Lindsey, 1961).

Ettinger and Suter (1970) reported that there was no sex predilection for *Dirofilaria immitis* infection, however a 3:1 male : female ratio was determined in dogs with microfilariae of *Dipetalonema reconditum*.

Falls and Platt (1982), Amer (1986) and Ananda and D'souza (2007) observed high infestation rates in male dogs than females and this might be due to hormonal effect on susceptibility of dogs to infection.

According to Martin and Collins (1985) male dogs were found more frequently infected with *Dirofilaria immitis* and less frequently with *Dipetalonema reconditum* than females in a survey conducted to study the prevalence of microfilariosis in Grey hounds of Australia.

Suprabha and Devada (2003) observed slightly higher prevalence in male dogs (54.5%) than female (45.5%) dogs.

Jabina and Ajith (2005) stated that the male : female ratio of dogs affected with microfilariosis was found to be 7:3.

Hashem and Badawy (2008) recorded high infestation rate in male dogs (85.7%) than females (14.3%).

Miterpakova *et al*. (2008) noticed that male dogs with long hair were significantly more frequently infected when compared with females and short hair breeds.

2.2.3. Breed

Suprabha and Devada (2003) noticed no breed predilection in dogs infected with *Dirofilaria repens.*

Ananda and D'souza (2007) observed highest infection rates in non descript breed followed by other breeds and the lowest observed in Labrador breeds.

2.2.4. Prevalence

Valsala and Bhaskaran (1974) stated that the incidence of canine filariosis was 13.04 percent in Calicut.

Dandapat *et al.* (1985) observed that the incidence of testicular hydrocele in humans due to filariasis was approximately 73 per cent in India.

According to Martin and Collins (1985) the prevalence of *Dirofilaria immitis* and *Dipetalonema reconditum* microfilariae in grey hounds of Australia was 10.9% and 3.6% respectively and recorded higher prevalence in summer than in winter.

Saseendranath *et al.* (1986) recorded an incidence of 24.2 per cent canine dirofilariosis in Trichur during a period of 6 months.

Phantana *et al.* (1995) reported that the prevalence of *Brugia malayi* in domestic cats living in the endemic area of Southern Thailand was estimated to be 4.3 per cent.

According to Radhika (1997) the prevalence of microfilariosis in dogs in Trichur was 7.59 per cent and was found to be higher during the summer season.

The filarial nematode *Brugia malayi* has been found in domestic cats in endemic areas in Southern Thailand with a prevalence ranging from 1.59 to 3.01% (Filariasis Division, 2001) and 0.63% and 2.4% in humans and cats respectively (Filariasis Division, 2002) and reduced to zero in both humans (Filariasis Division, 2003) and cats (Nuchprayoon *et al.*, 2006) and opined that this finding in the studied area could imply the effectiveness of the filariasis control program.

Chansiri *et al.* (2002) surveyed 53 feline blood samples from the endemic area of Surat Thani and Narathiwat, Southern provinces of Thailand and found that 15 of the domestic cats were infected with *Brugia malayi* suggesting the role

of domestic cats as animal reservoir for *Brugia malayi* in endemic areas of Thailand.

Cancrini *et al.* (2003) reported that the highest prevalence of 84.6 percent *Dirofilaria repens* microfilariosis in stray dogs in Spain.

Suprabha and Devada (2003) stated that 33 out of 310 dogs screened for microfilariosis by wet blood mount were found positive indicating a prevalence rate of 10.6 per cent in Trivandrum district.

According to Kobasa *et al*. (2004) the prevalence of human filarial parasite, *Brugia malayi* in naturally infected cats in Narathiwat province, Thailand was 8.51 per cent.

Sabu *et al.* (2005) reported 7 per cent incidence of canine dirofilariosis in Trichur due to *Dirofilaria repens.*

Ananda and D'souza (2007) opined that the prevalence of dirofilariosis with *Dirofilaria repens* in and around Mangalore city was 38.09 percent.

2.2.5. Transmission

Webber and Hawking (1955) reported that the prepatent period of *Dirofilaria repens* is 6 to 9 months.

Guptavinij *et al.* (1971a) conducted studies on subperiodic *Brugia malayi* in Thailand and opined that primary vectors for nocturnally subperiodic type were *Mansonia uniformis* and *Mansonia bonnae* where as *Coquilletidia crassipes* acts as important vectors for diurnally subperiodic types.

Guptavinij *et al.* (1971a, b) revealed that infection could be transmitted from human to cat and probably from infected cats to man (Lek-Uthai, 2004), but the periodicity changed from nocturnally periodic to nocturnally subperiodic.

Dondero *et al.* (1972) opined that the nocturnally sub-periodic form of *Brugia malayi* was zoonotic and that could infect both domestic and wild animals.

Rakai *et al.* (1974) opined that *Aedes polynesiensis, Aedes pseudoscutellaris, Aedes vigilax* and *Ochlerotatus niveus* were the principal vectors of the diurnally subperiodic form of *Wuchereria bancrofti* in the Nicobar group of Islands, India.

Farnell and Faulkner (1978) stated that *Dipetalonema reconditum* was transmitted by fleas, *Ctenocephalides felis* or *Ctenocephalides canis* and develop into adult by 10 weeks which live in the subcutaneous tissue of dogs.

Korkejian and Edeson (1978) reported a nocturnal periodicity of microfilariae of *Dipetalonema reconditum* in the peripheral blood.

Dissanaike (1979) reported domestic cats and monkeys as the animal reservoir host for subperiodic *Brugia malayi* in Thailand. The authors opined that control of brugian filariasis would be complicated because animal-to-human transmission continues even after the infection in humans has been greatly reduced and therefore in addition to chemotherapy and vector control, a successful lymphatic filariasis programme should also consider the control of reservoir hosts.

Bobade *et al.* (1981) opined that the microfilaraemia due to *Dipetalonema reconditum* exhibited a nocturnal cyclic variation with the peak level between 04.00 (4.00 a.m.) and 05.00 hours (5.00 a.m.) and the lowest level between 12.00 (12.00 noon) and 14.00 hours (2.00 p.m.).

Mak *et al.* (1982) studied on epidemiology of subperiodic *Brugia malayi* in an endemic area in Malaysia and opined that the unsatisfactory results with mass chemotherapeutic control was attributed to the zoonotic transmission of subperiodic *Brugia malayi* from non human primates with a mean infection rate of 76.3%.

WHO (1984) opined that periodic strains of *Brugia* species and *Wuchereria bancrofti* almost exclusively infect human beings where as subperiodic strains of *Brugia malayi* commonly infect domestic cats and various wild carnivores, from which transmission to human beings occurred through diurnally active *Mansonia* species.

Thammapaolo (1993) suggested that *Brugia malayi* could infect both humans and animals but *Brugia pahangi* can only infect animals, especially domestic cats.

Phantana *et al.* (1995) opined that the increasing incidence of filariasis in human beings in Southern Thailand might be due to the transmission of *Brugia malayi* from domestic cats to humans *via* a mosquito vector.

Arunachalam *et al.* (1996) opined that the vector for brugian and bancroftian species were *Mansonioides* species and *culex quinquefasciatus* respectively in Kerala.

Harrus *et al.* (1999) and Tarello (2000) suggested trans-placental transmission for *Dirofilaria repens.*

Kobasa *et al.* (2004) reported that the highly endemic status of human brugian filariasis in Narathiwat province, Thailand together with the high prevalence of *Brugia malayi* in cats there, emphasized the role of cats as a reservoir host of *Brugia malayi*.

Lee *et al.* (2007) revealed that *Dirofilaria immitis* and *Dirofilaria repens* were transmitted by mosquitoes belonging to several genera *viz., Anopheles, Aedes, Ochlerotatus, Culex, Armigeres* and *Mansonia* in Korea. Factors affecting transmission included mosquito population densities, mosquito species present, mosquito fecundity, favourable environmental temperatures and the availability of reservoir hosts.

Hashem and Badawy (2008) stated that the microfiariae of *Dipetalonema reconditum* showed a nocturnal periodicity with a significant increase in the number of circulating microfilariae in the peripheral blood towards the evening and peaked between 6 to 9 PM.

2.3. PATHOGENESIS

2.3.1. Canine Microfilaria

2.3.1.1. Dirofilaria repens

Schwan *et al.* (2000) reported acute liver failure in a cat with *Dirofilaria repens* infection.

Tarello (2000) reported pruritic dermatitis associated with *Dirofilaria repens* microfilaraemia in 19 cats from Central Italy and the dermatological lesions included alopecia, erythema, papulae, crusting and lichenification.

Mallick and Ittyerah (2003) reported subcutaneous dirofilariasis due to *Dirofilaria repens* in the eyelid of a 14 year old girl.

Tarello (2003) diagnosed dermatitis associated with *Dirofilaria repens* microfilaraemiae in a four year old Pyrenean Mountain dog from Rome and observed lesions all over the body with multifocal alopecia, erythema on head, neck and hind limbs, hyperkeratosis on lumbosacral region , papulae on abdomen and hind limbs.

2.3.1.2. Dirofilaria immitis

Morizon and Wright (1976) investigated the immunopathological aspects of canine renal disease and revealed that development of immunity against an infectious agent is not always beneficial to the host and some times resulted in deposition of immune complexes in the glomeruli which in turn stimulated chronic form of glomerulonephritis.

Shirota *et al.* (1979) noticed chronic interstitial nephritis in dogs infected with *Dirofilaria immitis*.

Rawlings (1986) opined that canine heart worm disease affected many organs including lung, heart, liver and kidneys. The author also reported renal amyloidosis in dirofilariosis.

Calvert (1987) noticed glomerulopathy, hepatic encephalopathy and haemoglobinuria in canine heartworm disease.

Sutton (1988) described immune mediated glomerulonephropathy in dogs infested with *Dirofilaria immitis* while Grauer *et al.* (1989) observed glomerulosclerosis in experimentally induced *Dirofilaria immitis* in dogs.

Rawlings and Calvert (1989) reported that the adult worms of *Dirofilaria immitis* residing in the right ventricle of the heart and the pulmonary arteries mechanically damage the endothelium eliciting an inflammatory reaction resulted in villous arteritis and consequently functional and morphological changes in the pulmonary tissues.

Mozos *et al.* (1992) described cutaneous lesions associated with canine heartworm infection by analyzing the clinical and histopathological features of five cases of chronic pruritic dermatitis in dogs infected with *Dirofilaria immitis*. Clinically, the cutaneous syndrome was characterized by a papulo-nodular and/or ulcerative non-specific dermatitis, responsive to antiparasitic treatment.

Calvert and Rawlings (1993) opined that the venacaval syndrome developed when number of adult worms of *Dirofilaria immitis* exceed hundred and occupy the right ventricle, right atrium and venacavae.

Polizopoulou *et al.* (2000) reported right sided congestive heart failure and caval syndrome in dogs naturally infected with *Dirofilaria immitis* in northern Greece and opined that caval syndrome was characterized by intravascular haemlysis, disseminated intravascular coagulation (DIC) and shock.

Grauer (2005) stated that canine dirofilariasis with *Dirofilaria immitis* resulted an immune complex mediated glomerulonephritis with persistant proteinuria. Persistant proteinuria, being a marker of renal disease was associated with progressive glomerular and tubulointerstitial lesions resulting in loss of nephrons.

2.3.1.3. Brugia pahangi

Malone and Thompson (1975) studied macroscopic pathology of *Brugia pahang* by inoculating infective larvae *via* subcutaneous route in golden hamsters and the gross lymphatic pathologic lesions consisted of moderate to marked dilation of lymphatic vessels, enlargement of regional lymph nodes, and numerous lymphothrombi and emboli. Macroscopic changes were most consistent and severe in the lymphatic vessels of the testes, epididymis, and spermatic cord and less frequently in the afferent or efferent vessels of various regional lymph nodes.

2.3.2. Human Microfilariae

Spry (1981) assessed patients with tropical filarial eosinophilia and suggested that blood eosinophils were induced to release their granule constituents into the circulation which may interact with microfilariae, and led to some of the clinical features of chronic filarial infections.

Dasgupta *et al*. (1984) stated that the clinico-pathological manifestations associated with human filariasis were fever, lymphangitis, lymphadenitis, lymphoedema, hydrocoele, elephantiasis and chyluria

Neilson (1989) opined that renal abnormalities in chronic filariasis included affections of both glomerular and tubulointerstitial compartments. This reflected parallel induction of glomerular and tubulointerstitial injury by parainfectious mechanisms or development of secondary tubulointerstitial disease in the course of glomerulonephritis.

Rath *et al.* (1991) observed that tubular degeneration was found in renal biopsy tissue from *Wuchereria bancrofti* infected patients with proteinuria.

Dreyer *et al.* (1992) found an elevated proteinuria in 20% of *Wuchereria bancrofti* microfilaraemic patients and suggested renal pathology with a typical tubular type of proteinuria associated with lymphatic filariasis.

According to WHO (1993) 'renal abnormalities in microfilaraemic individuals' were 'probably a form of nephritis related to parasite antigen-specific immune complexes.

Crandall *et al.* (1994) inoculated larvae of *Brugia rnalayi* in ferrets and opined that the principal pathologic response was chronic lymphangitis including thrombolymphangitis and greatly dilated dermal lymphatics in oedematous limbs.

Renal disease appears to be a common event in Brugian filariasis, involving both the tubular and glomerular compartment of the kidney. Its pathogenesis is obviously complex and not only immune complex-mediated (Langharnrner *et al.*, 1997).

Kemp and Roberts (2001) opined that chronic disease may led to chronic obstruction of lymph and serous fluid resulting in chronic, permanent, and disabling elephantiasis of the lower extremities or testes, and to a lesser extent, arms, breasts, labia, and penis. Chyluria results from chyle in the urine caused by obstruction between the intestinal lymphatics and the thoracic duct leading to rupture of renal lymphatics into renal tubules. Chyluria is often associated with back pain.

Taylor *et al.* (2001) confirmed the involvement of an endosymbiotic bacteria *Wolbachia* in the pathogenesis of acute inflammatory pathology associated with lymphatic filariasis.

Pathology associated with human filariasis was caused by adult parasite secretions, inflammatory reactions associated with the death of adult worms, secondary infections or simple failure of collateral lymphatic vessels resulting in chronic obstruction of lymphatic vessels which inturn results in filarial fevers, chronic lymphedema/elephantiasis, chyluria, and urogenital manifestations in men, such as hydrocele and elephantiasis of the penis or scrotum (Ottesen, 2006).

2.4. CLINICAL SIGNS

2.4.1. Canine Microfilaria

2.4.1.1. Dirofilaria repens

Joseph *et al.* (1976) suggested that *Dirofilaria Conjunctivae* (identical to *Dirofilaria repens* in dogs) caused conjunctivitis in human beings.

Kamalu (1991) opined that canine *Dirofilaria repens* infection had been associated with non specific skin changes like swelling and hyperpigmentation.

Bredal *et al.* (1998) reported subcutaneous granuloma on the chest of a dog from Norway due to occult infestation with an adult female *Dirofilaria repens.*

Tarello (2000) opined that the clinical signs of canine dirofilariasis due to *Dirofilaria repens* consisted of poor appetite, conjunctivitis, itching, cutaneous nodules and erythema.

Tarello (2002) reported pruritic dermatitis characterized by the presence of erythema, papules, focal or multifocal alopecia, crusting and nodules in 28 dogs with *Dirofilaria repens* microfilariae infestation in an endemic area in north-west Italy.

Jabina and Ajith (2005) observed off-feed, fever, congested mucous membrane, vomiting and oedema of hind limbs and scrotum as major symptoms of *Dirofilaria repens* microfilaraemic dogs.

Manuali *et al.* (2005) reported an unusual case of dirofilariasis with *Dirofilaria repens* in nipple discharge of a 6-yr-old Bernese female dog presented with a breast lump in central Italy.

2.4.1.2. Dirofilaria immitis

Jackson (1969) stated that principal signs associated with vena caval syndrome in heartworm disease consisted of sudden weakness, anorexia, haemoglobinuria and bilirubinuria.

Calvert (1987) reported that hypersensitivity reactions to microfilariae were manifested as dyspnoea, coughing, crackles, eosinophilia and basophilia.

Mozos *et al.* (1992) noticed a cutaneous syndrome characterized by a papulo-nodular and/or ulcerative non-specific dermatitis associated with canine heart worm infection.

Clinical signs associated with caval syndrome included abdominal effusion (ascites), exaggerated jugular pulses, anorexia, hemoglobinuria (considered pathognomonic for caval syndrome) and acute respiratory distress. Physical examination findings included pale mucous membrane, prolonged capillary refill time, weak femoral pulses, hepatosplenomegaly, heart murmur secondary to tricuspid insufficiency, and a cardiac gallop (Strickland, 1998).

Atkins (2005) observed weight loss, exercise in tolerance, lethargy, poor body condition, cough, dyspnea, cyanosis, hemoptysis, syncope, and abdominal distention/ascites in dogs with heartworm disease.

According to Ghulam *et al.* (2005) the symptoms of canine dirofilariasis included exercise intolerance, fever, chronic cough, dyspnoea, progressive weight loss and voiding of concentrated urine.

2.4.1.3. Dipetalonema reconditum

Bobade *et al.* (1981) stated that the main clinical findings associated with microfilariae of *Dipetalonema reconditum* in dogs were dermatosis and emaciation.

Hargis *et al.* (1999) diagnosed dermatitis associated with microfilariae of *Dipetalonema sp*. in 10 dogs from the western United States. Clinically, the lesions were single or multiple papules and plaques with alopecia, scarring, erythema, ulceration or crusting.

2.4.2. Human Microfilaria

Dasgupta *et al*. (1984) stated that the clinico-pathological manifestations associated with human filariasis were fever, lymphangitis, lymphadenitis, lymphoedema, hydrocoele, elephantiasis and chyluria .

According to Nutman and Weller (1998) the clinical manifestations of lymphatic filariasis may range from asymptomatic microfilariasis to hydrocele, lymphangitis, lymphadenitis with high-grade fever (filarial fever), and lymphatic obstruction.

Clinical manifestations associated with lymphatic filariasis in human patients were filarial fever, chronic lymphedema/elephantiasis, chyluria, and urogenital manifestations in men, such as hydrocele and elephantiasis of the penis or scrotum (Ottesen, 2006).

Rohela *et al.* (2006) reported conjunctivitis and palpebral swelling in a 3 year old child from Malaysia caused by adult *Brugia malayi* which was retrieved from beneath the conjunctiva. Giemsa stained blood smear was positive for microfilariae of *Brugia malayi* and later it was confirmed by *Brugia* Rapid test.

2.5. DIAGNOSIS

2.5.1. Wet Film Examination

Saseendranath *et al.* (1986) studied the incidence of canine microfilariosis in Trichur district by wet film examination of peripheral blood.

Radhika (1997) observed three distinct patterns of motility of *Dirofilaria repens* microfilariae in wet blood film *viz.*, wriggling , progressively forward moving and wriggling cum progressively forward moving type.

Courtney and Zeng (2001) opined that eventhough a variety of tests could be used for diagnosing canine dirofilariosis , the detection of microfilariae in wet blood film remained the best method of ascertaining that the dog was infected.

Ghulam *et al.* (2005) revealed that microscopic examination of wet blood film positive for *Dirofilaria immitis* microfilariae showed numerous microfilariae with non progressive bunting movement.

Ananda and D'souza (2007) screened dogs for microfilariosis in Karnataka state by wet film examination of peripheral blood and positive cases revealed wriggling and progressively forward type of motility.

Hashem and Badawy (2008) stated that in wet blood film, the microfilariae appeared as a snake like with a rapidly forward movement across the microscopic field.

2.5.2. Giemsa Staining

2.5.2.1. Canine Microfilaria

2.5.2.1.1. Dirofilaria repens

According to Radhika (1997) the microfilariae of *Dirofilaria repens* were sheathless with a blunt head and a long tapering tail and the cuticle appeared to be striated in higher magnifications. Nerve ring and excretory cell at the excretory pore region of the microfilariae could be well appreciated. The nuclear column cells did not extended up to the tip of the tail.

Suprabha and Devada (2003) stated that microfilariae of *Dirofilaria repens* were sheathless, with a tapering head and long pointed tail.

Ananda *et al.* (2006) differentiated species of microfilariae based on morphological characters in giemsa stained smears and opined that the microfilariae of *Dirofilaria repens* were unsheathed with blunt head and a tapering tail and the cuticle appeared striated.

2.5.2.1.2. Dirofilaria immitis

Calvert and Rawlings (1993) revealed that the microfilariae of *Dirofilaria immitis* were straight with a tapered head and a straight tail.

Nuchprayoon *et al.* (2006) diagnosed *Dirofilaria immitis* infection in cats in Thailand and opined that the microfilariae of *Dirofilaria immitis* were unsheathed with one nucleus in the cephalic space.

Niwetpathomwat *et al.* (2007) identified *Dirofilaria immitis* microfilariae based on morphologic peculiarities in giemsa stained blood smears and it appeared as non sheathed with a tapered head and a straight tail.

2.5.2.1.3. Dipetalonema reconditum

According to Calvert and Rawlings (1993) microfliariae of *Dipetalonema reconditum* were shorter, curved tail shaped like a button hook and had a cephalic hook.

Ananda *et al.* (2006) demonstrated the microfliariae of *Dipetalonema reconditum* as unsheathed, curved tail with cephalic hook in the anterior end. The cuticle was unstriated in giemsa stained smears.

Hashem and Badawy (2008) identified *Dipetalonema reconditum* microfilariae which appeared coiled or twisted to a varying degree with no anterior nuclei and a posterior hooked tail end in giemsa stained smears from stray dogs.

2.5.2.1.4. Brugia pahangi

Nuchprayoon *et al.* (2006) detected *Brugia pahangi* microfilariae in domestic cats in Thailand and revealed that giemsa staining of this parasite showed clear sheathed microfilariae with 2 terminal nuclei.

2.5.2.2. Human Microfilaria

2.5.2.2.1. Brugia malayi

Kanjanopas *et al.* (2001) tentatively identified *Brugia malayi* microfilaria in a giemsa stained thick blood smear from domestic cats in Thailand and later confirmed as *Brugia malayi* by micrometry of the parasite in stained smears and histochemical staining techniques.

Kobasa *et al.* (2004) assessed the dimensions of *Brugia malayi* microfilariae in Giemsa stained smears under a compound microscope using a camera lucida drawing. Microfilariae of *Brugia malayi* were smaller in size, possess secondary kinks, and the nuclei extend upto the tip of the tail. There were

two discrete nuclei-one at the extreme tip of the tail and the other midway between the tip and the posterior column of nuclei.

2.5.2.2.2. Wuchereria bancrofti

Maheshwari *et al.* (2007) identified *Wuchereria bancrofti* microfilariae in a thyroid aspirate smear based on staining characters. The microfilariae were characterised by a sheath which is much longer than the larval body, discrete nuclei seen throughout with the tail tip being free of nuclei and a short cephalic space.

2.5.3. Histochemical Differentiation

2.5.3.1. Canine Microfilariae

Chalifoux and Hunt (1971) evaluated the acid phosphatase enzyme activity of canine microfilariae using Naphthol AS-TR Phosphate method and suggested that *Dirofilaria immitis* and *Dipetalonema reconditum* could be distinctly and accurately distinguished by this technique. Enzyme activity was restricted to two distinct zones in *Dirofilaria immitis* where as in *Dipetalonema reconditum* enzyme activity was uniformly distributed through out the organism.

Balbo and Abate (1972) differentiated microfilariae of *Dirofilaria immitis, Dirofilaria repens* and *Dipetalonema reconditum.* In *Dirofilaria immitis,* the acid phosphatase enzyme activity was restricted to excretory and anal vesicles. In *Dirofilaria repens,* the enzyme activity detected only at the anal vesicles. The acid phosphatase activity of *Dipetalonema reconditum* was distributed uniformly through out the body.

Kelly (1973) and Whitlock (1978) opined that acid phosphatase staining technique was the most accurate method for the speciation of microfilariae.

Acevedo *et al.* (1981) described the acid phosphatase staining pattern of *Dirofilaria immitis* and *Dipetalonema reconditum*. *Dipetalonema reconditum* shows a more uniform staining with a slightly lighter area from the cephalic end

to the excretory vesicle where as *Dirofilaria immitis* shows enzyme activity at the excretory and anal vesicles.

Ortega-Mora *et al.* (1989) studied the distribution of acid phosphatase enzyme activity in *Dipetalonema dracunculoides* microfilariae in dogs and found that enzyme activity was restricted to excretory pore, central body region and anal vesicle.

Peribanez *et al.* (2001) suggested an alternate technique for histochemical staining using a commercial test kit of Naphthol AS-OL (Leucognost-SP) and compared this technique with the standard Barka procedure and found that the observed areas of acid phosphatase activity coincided for each species of filarid worm, independent of the staining procedure.

Lee *et al.* (2004) utilized a commercial acid phosphatase staining kit (Leucognost–SP®, Merck, Germany) for the differentiation and species identification of canine microfilariae. The authors compared acid phosphatase staining with polymerase chain reaction (PCR) analysis for the detection of *Dirofilaria repens* infection in dogs in Korea and opined that all samples found to be positive by the acid phosphatase staining were also found to be positive by PCR analysis.

Radhika and Subramaniam (2005) and Ananda *et al.* (2006) opined that histohemical differentiation was found to be the most accurate, specific and reliable staining technique and described two patterns of acid phosphatase enzyme activity of *Dirofilaria repens* microfilariae, depending upon pattern of movement. The wriggling type showed acid phosphatase activity at the anal pore region only where as progressively forward moving type showed activity at the anal pore and central body region.

Toparlak *et al.* (2005) identified *Dipetalonema reconditum* in dogs of Turkey by Naphthol AS-TR Phosphate technique and found that *Dipetalonema*

reconditum exhibited uniform enzyme activity through out the body with higher enzyme activity noticed between excretory and anal pore.

Kesdangsakonwut *et al.* (2006) reported *Dirofilaria immitis* in captive asiatic jackal in Dusit Zoo in Bangkok and opined that the acid phosphatase enzyme activity of *Dirofilaria immitis* was restricted to excretory and anal vesicles and all samples positive for histochemical staining was found to be positive for *Dirofilaria immitis* antigen detection test (witness HW test)

Miterpakova *et al.* (2008) identified dirofilariosis in Slovakia using histochemical staining based on the different somatic distribution of acid phosphatase activity using commercial test kit (Leucognost-SP®, Merck, Darmstadt, Germany).

2.5.3.2. Human Microfilariae

Terwedow and Huff (1976) reported that the microfilariae of *Wuchereria bancrofti* exhibited acid phosphatase enzyme activity at the excretory vesicle, innenkorper and anal vesicle.

Omar (1977) demonstrated acid phosphatase activity in the larval stages of *Wuchereria bancrofti*, *Brugia malayi*, *Brugia pahangi* and *Dirofilaria immitis.*

Histochemical staining technique provides sufficiently characteristic and consistent results for the differentiation of even closely related species. Acid phosphatase activity of *Brugia malayi* microfilaria demonstrated intense positive sites at amphids, excretory and anal vesicles and phasmids with very diffuse activity in other parts where as *Brugia pahangi* showed intense activity along the entire body (Yen and Mak, 1978).

Fan *et al.* (1985) reported that the microfilariae of both species of Brugia in thick blood films stained with Naphthol AS-TR Phosphate showed that the excretory and anal pores of subperiodic *Brugia malayi* microfilariae exhibited acid phosphatase activity and only a little activity was seen in other parts while

Brugia pahangi microfilariae showed heavy diffuse acid phosphatase enzyme activity along the entire length of the body.

Kanjanopas *et al.* (2001) studied on feline microfilariosis in Thailand and opined that the acid phosphatase activity of *Brugia malayi* microfilariae were restricted to amphids, excretory and anal vesicles and phasmids whereas *Brugia pahangi* exhibited intense uniform enzyme activity along the entire body.

Kobasa *et al.* (2004) observed intensely positive acid phosphatase activity at the amphids, excretory and anal vesicles and phasmids in *Brugia malayi* microfilariae from feline blood smears in Thailand.

2.5.4. Micrometry

2.5.4.1. Canine Microfilaria

2.5.4.1.1. Dirofilaria repens

Radhika (1997) reported *Dirofilaria repens* as the sole cause of canine microfilariosis in Trichur based on micrometry, morphology and histochemical staining characteristics of the parasite. The average length and width of *Dirofilaria repens* microfilariae were 285.131±8.16 µm and 6.0±0.2 µm respectively.

Suprabha and Devada (2003) revealed that the average length, width, distance of nerve ring and excretory pore from the anterior end of *Dirofilaria repens* microfilariae were found to be 165-180 µm , 5.76-7.2 µm, 35-45 µm and 50-54 µm respectively.

Soulsby (2005) described the micrometry of different species of microfilariae found in dogs and opined that the average length and percentage distance of nerve ring, excretory pore and anal pore from the anterior end of *Dirofilaria repens* microfilariae were 290±10 µm, 23, 30 and 70 percent respectively.

According to Ananda *et al.* (2006) the microfilariae of *Dirofilaria repens* measured an average length of 292.2 ± 1.267 µm and width of 5µm.

2.5.4.1.2. Dirofilaria immitis

Lindsey (1965) identified microfilariae of *Dirofilaria immitis* based on mean location of internal structures *viz.*, nerve ring, excretory cell, excretory pore,germ cells, anal pore and terminal nucleus. The average length and width of *Dirofilaria immitis* microfilariae were 314 (286-340) µm and 6.8 (6.1-7.2) µm respectively.

Acevedo *et al.* (1981) and Calvert and Rawlings (1993) suggested that the microfilariae of *Dirofilaria immitis* were 327.46 ± 2.36 µm in length.

Ghulam *et al.* (2005) observed that the microfilariae of *Dirofilaria immitis* measures 298-315 µm in length and 6.2-7 µm in width.

Soulsby (2005) stated that the average length and percentage distance of nerve ring, excretory pore and anal pore from the anterior end of *Dirofilaria immitis* microfilariae were 313 (307-322) µm, 23.8, 32.7 and 82% respectively.

2.5.4.1.3. Dipetalonema reconditum

According to Lindsey (1965) microfilariae of *Dipetalonema reconditum* measured an average length and width of 270 (258-292) µm and 5.2 (4.7-5.8) µm respectively.

Acevedo *et al.* (1981) opined that the length of *Dipetalonema reconditum* microfilariae processed by formalin fixation/Knott technique was found to be 262.09±3.36 µm.

According to Soulsby (2005) the average length and percentage distance of nerve ring, excretory pore and anal pore from the anterior end of *Dipetalonema* reconditum microfilariae were 270.6 (246-293) µm, 20.8, 21 and 89 respectively.

According to Ananda *et al.* (2006) the microfliariae of *Dipetalonema reconditum* measured an average of 260.5 ± 33.337µm in length and 5.14 ± 0.067µm in width.

Hashem and Badawy (2008) identified microfilariae of *Dipetalonema reconditum* based on micrometry and staining characters in Giemsa stained smears and the microfilarial length ranged from 250-260 µm and diameter varied from 3.5-4.5 µm. The anterior end of microfilariae were devoid of nuclei to a distance about 7-8 µm. The nerve ring and excretory pore located at an average distance of 30±0.68 µm and 42±0.84 µm from the anterior end respectively. The anal pore located at 65±0.98 µm from the tail end which showed mostly a hooked appearance.

2.5.4.1.4. Brugia pahangi

Sivanandam and Fredericks (1966) identified *Brugia pahangi* microfilariae based on length of innenkorper and revealed that the average and range of innenkorper length of *Brugia pahangi* microfilariae were 53.1(44-63) µm.

Kelly (1979) reviewed filaroid nematodes in dogs and opined that *Brugia pahangi* measured about 280µm.

2.5.4.2. Human Microfilaria

2.5.4.2.1. Brugia malayi

Sivanandam and Fredericks (1966) opined that the average and range of innenkorper length of *Brugia malayi* microfilariae was 30.7(24-34)µm.

Kanjanopas *et al.* (2001) identified *Brugia malayi* microfilariae in cats by morphometry and revealed that the average and range of body length, body width at the nerve ring and innenkorper length were 221.30 (189.54-242.19) µm, 4.51 (3.33-5.55) µm and 32.29 (21.06-42.12) µm respectively.

Kobasa *et al.* (2004) carried out morphometric measurement of 313 microfilariae of *Brugia malayi* from 21 infected cats revealed that the average and range of body length, width at the nerve ring and innenkorper length were 195.91 ± 18.92 (144.33-249.98) µm, 5.25 ± 0.85 (3.21-7.49) µm and 28.56 ± 6.08 (11.11-44.44) µm respectively.

Soulsby (2005) described the morphology and micrometry of different species of microfilariae found in dogs and human beings. The average length and percentage distance of nerve ring, excretory pore and anal pore from the anterior end of *Brugia malayi* microfilariae were 220±20 µm, 24.5, 35 and 83 respectively.

2.5.4.2. Wuchereria bancrofti

Bhandari *et al.* (2006) analysed morphological variations of *Wuchereria bancrofti* from different endemic regions *viz.*, Calicut, Wardha and Bhubaneshwar. Important parameters such as total length and width of microfilaria and the localization of different fixed points such as cephalic space, nerve ring , excretory pore , excretory cell and anal pore from anterior end were measured and fixed point ratios were calculated. The microfilariae collected from Wardha region were found to be shorter (275.2 + 2.89) in length when compared to microfilariae from Calicut (292.0 + 3.9). Similarly, the width of microfilaria from Wardha region was also less compared to that of microfilariae from other regions. Thus, these differences in the measured parameters of microfilariae indicated the existence of different strains of *W. bancrofti* in India.

2.5.5. Immunological Test

Wilkins and Ciurea (1985) recommended immunofluorescent antibody (IFA) test and enzyme linked immunosorbant assay (ELISA) for the detection of microfilarial antibodies.

Lalitha *et al.* (1998) opined that monoclonal antibody-based ELISA using filter strips may be used in day time and replace the existing routine night blood surveys in lymphatic filariasis endemic areas in India.

Snyder *et al.* (2000) evaluated performance of serologic tests used to detect heartworm infection in cats and opined that combining results from serum Ab and Ag tests achieved higher sensitivities than using serum Ab and Ag test results alone (ie, maximum sensitivities of 100% *vs* 89.5%, respectively), where as use of serum Ag and Ab test results alone achieved higher specificities compared with the use of a combination of serum Ab and Ag results (ie., maximum specificities of 99.4% *vs* 92.9%, respectively).

Ganesh *et al.* (2001) reported that dot blot ELISA using a 66KDa *Brugia malayi* microfilarial antigen was a simple and inexpensive assay for the diagnosis of bancroftian filariasis in endemic areas.

Wickremanayake *et al.* (2001) developed a dot-ELISA for detection of *Wuchereria bancrofti* infection in an endemic area using *Setaria digitata* (cattle filarial worm) antigens. This test could differentiate the endemic normals from the microfilaraemic asymptomatic individuals.

Rahmah *et al.* (2003) developed an antibody detection dipstick test, named Brugia Rapid (BR), that detects IgG_4 antibodies reactive to a recombinant *Brugia malayi* antigen (BmR1), appeared to be a promising tool for mapping and monitoring the areas endemic for brugian filariasis.

Baskar *et al.* (2004) developed a rapid flow-through immune filtration test using recombinant filarial antigen *WbSXP-1* for detecting IgG antibodies of brugian and bancroftian filariasis in human beings in endemic areas. The sensitivity of the test was found to be 90.8% with brugian and 91.4% with bancroftian filariasis.The test showed minimum reactivity with *Loa loa* and no reactivity with *Onchocerca* or other parasitic diseases. The authors also opined

that failure of detection of antibody in some patients with brugian and bancroftian filariasis may be due to the varying degree of chronicity of the disease.

Berdoulay *et al.* (2004) compared serological tests for the detection of natural heartworm infection in cats and concluded that combining results from antigen and antibody tests achieved greater sensitivity than using either test alone.

2.5.6. PCR and Gene Sequencing

Ransohoff *et al.* (1989) opined that DNA sequence analysis of genes encoding 5S rRNA in the human parasitic nematode *Brugia malayi* indicated a surprising degree of heterogenecity. This variation in coding sequence was not accompanied by corresponding heterogeneity in flanking regions which were highly conserved.

Favia *et al.* (1996, 1997), Baneth *et al.* (2002) and Mar *et al.* (2002) utilized polymerase chain reaction (PCR) technology to diagnose filarial infestations in dogs and human beings.

Sambrook and Russel (2001) described the standard phenol chloroform method for isolation of pure DNA from samples.

Chansiri *et al.* (2002) utilized trans spliced leader exon 1(SLX) gene and Hha1 repetitive region (HR) for identification of human *Brugia malayi* in domestic cats in endemic areas in Thailand. Upon PCR amplication of SLX gene, a single band of 294 bp PCR fragment was obtained from *Brugia malayi* where as two PCR fragments of 294 bp and 310 bp in size were obtained from *Brugia pahangi*. The PCR amplication of HR region of human and feline *Brugia malayi* microfilarial DNA's revealed a PCR fragment of 294 bp which on restriction fragment length polymorphism (RFLP) using Alu1 digestion generated two fragments of 224 bp and 70 bp. The authors opined that the identification of human and feline *Brugia malayi* microfilaria based on PCR amplification of SLX and PCR-RFLP of HR region revealed that they were identical.

Lee *et al.* (2004) compared acid phosphatase staining with the polymerase chain reaction analysis for the diagnosis of *Dirofilaria repens* infection in dogs in Korea and concluded that PCR analysis was more valuable for the diagnosis of *Dirofilaria repens* infection than acid phosphatase staining.

Rishniw *et al.* (2006) developed a simple molecular method of identifying and discriminating six species of microfilariae known to infect dogs by amplifying ribosomal DNA spacer sequences by polymerase chain reaction using common and species - specific primers. The pan-filarial primers (DIDR-F_1 and DIDR-R_1) were used to amplify products of 542, 578, 484 and 584 bp from microfilaraemic canine blood samples positive for *Dirofilaria immitis, Acanthocheilonema reconditum, Dirofilaria repens and Acanthocheilonema dracunculoides* respectively. They also amplified 615 and 664 bp products from DNA extracted from adult worms of *Brugia malayi* and *Brugia pahangi* respectively. The authors also amplified two additional filariae (onchocerca and mansonella) using the pan-filarial primers and suggested that the pan-filarial primer designed by them might be suitable for genotyping all members of the family *Onchocercidae*. The amplicons were cloned and sequenced to confirm the genotype of the filariae. The sequences obtained were compared with those already present in Gene Bank data base at the National Center for Biomedical Information and assigned accession numbers.

2.5.7. Ultrasonography

Walter *et al.* (1987) investigated ultrasonographic abnormalities associated with renal parenchymal diseases in dogs and revealed that kidneys with end-stage disease were often diffusely hyperechoic and have poor corticomedullary delineation and an irregular shape, small size and have decreased cortical thickness and irregular margination with thick capsules. Glomerulonephritis, glomerulosclerosis, chronic interstitial nephritis, amyloidosis, and nephrocalcinosis were associated with hyperechoic kidneys in end-stage disease.

Lamb (1990) reviewed abdominal ultrasonography in small animals and opined that normal hepatic parenchyma had a uniformly slightly coarse echotexture, in which the larger blood vessels and gall bladder were visible. Dilated hepatic veins draining into the caudal venacava were appreciated in case of hepatic congestion.

Wood and McCarthy (1990) revealed that ultrasonograph of the cortex of the normal canine kidney had a relatively uniform, fine speckled echotexture compared to which the medulla is markedly hypoechoic. Fine discontinuous echogenic lines at the corticomedullary junction, which represent the arcuate arteries, were normally observed.

Biller *et al.* (1992) opined that ultrasonographic investigation of canine kidney revealed medullary rim sign in association with chronic interstitial nephritis and acute tubular necrosis.

Lamb (1995) opined that hepatic lesions were recognizable ultrasonographically when they alter the morphology of the liver, its surface contour, parenchymal echogenicity or vasculature or the biliary tract.

According to Mantis and Lamb (2000) dogs with renal disease and medullary rim sign were likely to have other ultrasonographic signs of renal disease whereas finding medullary rim sign alone was more likely in dogs without renal dysfunction.

Raila *et al.* (2007) performed ultrasonographic examination of the abdomen in a 14-month-old bernese mountain dog with chronic renal failure and showed hyperechogenic kidneys, in which the renal cortex and medulla were not distinguishable.

Singh *et al.* (2007) stated that the ultrasonographic appearance of hepatic parenchyma revealed hypoechogenicity with increased visualization of many portal vein branches, which was seen as a starry sky appearance in dogs with

hepatitis. Diffusely hyperechoic and irregularly marginated liver could be appreciated in case of cirrhosis.

2.5.8. Electrocardiography

Calvert *et al.* (1986) opined that cardiac rhythm disturbances were not common in heart worm disease and right ventricular hypertrophy was seen only in dogs with severe pulmonary arterial disease.

Atkins (2005) opined that electrocardiography revealed arrhythmias, such as premature atrial or ventricular beats, and conduction abnormalities (right bundle branch block) in moderate to severe canine heart worm disease ,but not common unless cardiac enlargement was moderate to severe.

Jabina and Ajith (2005) recorded ECG changes associated with canine microfilariosis and it included deep Q and S waves in lead I, II and III and tall R wave and peaked T wave in lead ll, which indicated biventricular hypertrophy and myocardial hypoxia or hyperkalaemia in affected animals.

2.5.9. Radiography

Losonsky *et al.* (1983) reported that radiographic changes associated with heartworm disease include right ventricular enlargement, increased prominence of the main pulmonary artery segments, increased size and density of the pulmonary arteries, arterial tortuosity and pruning.

2.6. HAEMATOLOGICAL ANALYSIS

2.6.1. Haemogram

Sharma and Pachauri (1982) noticed a non significant reduction in haemoglobin, total erythrocyte count and volume of packed red cells (PCV) in dogs infected with *Dirofilaria repens.*

Anaemia in canine filariosis might be due to haemolysis as a result of destructive motility of microfilariae as suggested by Kitagawa *et al.* (1989).

Yousif *et al.* (1990) stated that decrease in erythrocyte production in microfilariosis were attributed to the damage caused by microfilariae to the liver tissues resulting in loss of blood and depression of erythropoesis.

Reifer *et al.* (2004) reported a significant macrocytic anaemia in dogs with mixed infection of *Dirofilaria immitis*, *Dipetalonema reconditum* and a third unidentified (mf^3) microfilariae.

Ananda and D'souza (2006) observed a non significant reduction in haemoglobin (12.61±0.14g/dl), total erythrocyte count (7.28±0.21millions/mm^3) and volume of packed red cells (PCV) in dogs infected with *Dirofilaria repens*

Anuchai *et al*. (2006) found moderate macrocytic anaemia in dogs with dirofilariosis, ehrlichiosis and babesiosis.

Niwetpathomwat *et al.* (2007) reported mild to moderate anaemia in microfilaraemic dogs with a significant decrease in the total erythrocyte count (5.7 ± 1.6 millions/ mm^3), haemoglobin (12.4 ± 6.7 g/dl) and volume of packed red cells (36.9 ± 10.6%).

Hashem and Badawy (2008) suggested that the blood cellular analysis of *Dipetalonema reconditum* infested dogs revealed a significant reduction in RBC (3.4 ± .12 millions/mm^3), haemoglobin (9.5 ± 1.12 g/dl) and PCV (28 ± 1.12%) values and increase in reticulocyte count, MCV, MCH with a decrease in MCHC suggesting the presence of regenerative anaemia of macrocytic hypochromic type.

2.6.2. Leucogram

Sharma and Pachauri (1982) noticed neutropenia, eosinophilia, lymphocytosis and a higher erythrocyte sedimentation rate (ESR) in dogs infected with *Dirofilaria immitis.*

Paltrinieri *et al.* (1998) opined that neutrophilic leucocytosis in dogs with dirofilariosis might be due to increased phagocytic removal of tissue breakdown products of microfilariae or inflammatory response to the parasite.

Feldman *et al.* (2000) opined that eosinophilia was due to sensitivity to the foreign protein of a parasite which might be a part of an immune phenomenon while Ananda and D'souza (2006) observed leucocytosis (15.86 ± 0.42 thousands/mm^3), eosinophilia, lymphocytosis and neutropenia in dogs with dirofilariosis.

Niwetpathomwat *et al.* (2007) and Hashem and Badawy (2008) observed marked leucocytosis, moderate to marked neutrophilia, eosinophilia and monocytosis in microfilaraemic dogs.

2.6.3. Platelet

Thrombocytopenia was reported in dogs infected with *Dirofilaria immitis* as a result of immune mediated platelet destruction (Waner *et al.,* 1995; Anuchai *et al.,* 2006 and Niwetpathomwat *et al.,* 2007).

Ananda and D'souza (2006) observed a marked reduction in platelet count (191.20 ± 15.14 thousands/ mm^3) in *Dirofilaria repens* microfilaraemic dogs when compared to normal dogs.

Naik-Mathuria *et al.* (2008) observed thrombocytopenia in a man with pulmonary dirofilariasis caused by *Dirofilaria repens.*

2.7. SERUM BIOCHEMICAL ANALYSIS

2.7.1. Alanine amino transferase (ALT)

Calvert and Rawlings (1993) observed increase in liver specific enzyme alanine amino transferase in dogs with heart worm disease.

Ananda and D'souza (2006) attributed high activity of alanine amino transferase up to an average of 51.24±1.89 IU/L in dirofilariasis affected animals due to localization of a large number of circulating microfilaria in the hepatic portal vein.

Niwetpathomwat *et al.* (2007) reported that elevation of serum alanine amino transferase (82±76IU/L) was considered one of the most common serum biochemical abnormalities in microfilaraemic dogs

Hashem and Badawy (2008) revealed that a significant increase in alanine amino transferase (120.66±4.30IU/L) in dogs infected with *Dipetalonema reconditum* microfilariae indicating hepatic dysfunction associated with the disease.

2.7.2. Aspartate Amino Transferase (AST)

Ananda and D'souza (2006) observed high serum activity of aspartate amino transferase (55.18±0.65IU/L) in microfilaraemic dogs when compared to normal dogs.

Niwetpathomwat *et al*. (2007) reported an elevation of serum aspartate amino transferase activity of 50±38 IU/L in dogs infected with dirofilariosis in Thailand.

According to Hashem and Badawy (2008) dogs infected with *Dipetalonema reconditum* showed a significant increase (150 ± 5.33 IU/L) in serum aspartate amino transferase.

2.7.3. Alkaline Phosphatase (ALP)

According to Calvert and Rawlings (1993) heart worm disease in dogs was associated with an increase in serum alkaline phosphatase enzyme.

Niwetpathomwat *et al.* (2007) assessed serum alkaline phosphatase activity in canine microfilariosis and observed an increased serum levels of 311 ± 299 IU/L in infected dogs.

2.7.4. Total protein, Albumin, Globulin and AG ratio

Moustafa *et al.* (1991) suggested that hyperproteinemia in filariasis might be due to an increase in globulin concentration in response to parasitic antigens or due to release of haemoglobin from lysed erythrocytes.

Kitagawa *et al*. (1998) observed hypoalbuminemia in dogs with heartworm disease and opined that the hypoalbuminemia corresponds to the degenerative changes in haemoparasitized organs mainly liver.

Hyperproteinemia (8.95 ± 0.16 g/dl), hypoalbuminemia (3 ± 0.04 g/dl) and hyperglobulinemia (5.95 ± 0.32 g/dl) with a reduced albumin globulin ratio were reported in *Dipetalonema reconditum* infected dogs (Hashem and Badawy, 2008).

Naik-Mathuria *et al.* (2008) observed hypoalbunminemia in human pulmonary dirofilariasis.

2.7.5. Blood Urea Nitrogen

Anuchai *et al.* (2006) observed significantly higher blood urea nitrogen in dogs affected with dirofilariasis.

Niwetpathomwat *et al.* (2007) evaluated the renal function in heartworm disease and revealed that an increase in blood urea nitrogen of 46.5±63.7 mg/dl in these cases were associated with damages of renal origin.

Hashem and Badawy (2008) opined that a significant increase of blood urea nitrogen (73.92±2.04 mg/dl) in dogs infected with *Dipetalonema reconditum* suggesting renal involvement of the condition.

2.7.6. Serum Creatinine

Ananda and D'souza (2006) observed a significant increase in serum creatinine (1.47 ± 0.05 mg/dl) in dirofilariasis affected dogs.

Anuchai *et al.* (2006) observed significantly higher serum creatinine levels in dirofilariasis affected dogs than non infected one and this might be due to severe kidney dysfunction and intravascular haemolysis.

Niwetpathomwat *et al.* (2007) revealed a significantly increased serum creatinine level up to an average of 2.2±4 mg/dl in canine heart worm disease.

Hashem and Badawy (2008) evaluated serum creatinine (1.5±0.02 mg/dl) levels in dogs infected with *Dipetalonema reconditum* and suggested a significant increase in these animals.

2.8. URINALYSIS

Center *et al.* (1985) evaluated 24 hour urine protein creatinine ratio (UPC) in dogs with protein losing nephropathies in comparison with healthy controls and opined that the average UPC in dogs with glomerulonephritis, amyloidosis and chronic interstitial nephritis were 5.73, 22.50 and 2.89 respectively when compared to a UPC of less than 0.2 in healthy dogs. High UPC of more than 10 in dogs was suggestive of severe glomerulonephritis and amyloidosis.

According to Uechi *et al.* (1994) urine enzyme indices in dogs revealed no significant differences between males and females.

Langharnrner *et al.* (1997) demonstrated and characterized renal disorders in asymptomatic microfilaraemic carriers, patients with filarial fever, lymphangitis, lymphoedemas and elephantiasis from *Brugia malayi* endemic areas of Kerala. Urinalysis of all infected patients showed significantly higher NAG activity than endemic controls. The presence of kidney disease was clearly indicated by a significantly elevated proteinuria in all infected patients compared to endemic and nonendemic control groups. Tubular disorders were clearly

predominating in asymptomatic microfilaraemic carriers while a glomerular type of proteinuria was observed in chronically ill individuals. Patients with filarial fever showed purely tubular disorders, but in other cases with lymphangitis alone exhibited a mixed type of proteinuria.

Clemo (1998) stated that N-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyl transpeptidase (GGT) were renal tubular enzymes which were primarily located in the lysosomes and brush border respectively, of the proximal convoluted tubule and these enzymes were released into the urine as a result of renal tubular injury.

According to Atkins *et al.* (2000) urinalysis of cats with dirofilariasis revealed proteinuria with reduced specific gravity.

Bazzi *et al.* (2002) opined that urine NAG excretion was commonly used as a marker of tubular and glomerular injury in human medicine

Sato *et al.* (2002) indicated that dogs with chronic renal failure showed an increased urine NAG index prior to elevation of blood urea nitrogen and creatinine concentrations. So urinary NAG index was considered more sensitive for early detection of renal damage than serum creatinine level. The authors also opined that measurement of NAG and its isoenzymes revealed more information about tubular damage at an early stage in cats with chronic renal failure.

Forterre *et al.* (2004) suggested that measurement of total urinary proteins in dogs by urinary dipstick was a typical method for assessing the presence of potentially serious renal disorders. The average urine specific gravity, urine protein, urine creatinine and urine protein creatinine (upc) of urine samples obtained from 12 dogs with chronic renal disease were 1.01, 77.2 mg/dl, 44 mg/dl and 1.62 respectively.

Langston (2004) opined that microalbuminuria is an albumin concentration beneath the limit of detection by standard dipsticks and it is an important indicator of early renal damage. A new dipstick test had been

developed to measure microalbuminuria in cats based on ELISA utilizing a feline albumin antibody.

Cowgill and Francey (2005) revealed that measurement of activity of renal tubular enzymes like NAG, GGT, alkaline phosphatase *etc* were more sensitive for detection of acute renal damage than the current standard veterinary diagnostic tests like assessment of serum creatinine, blood urea nitrogen (BUN) and urine specific gravity. These urinary enzymes shows detectable increase with acute tubular damage prior to detectable increases in BUN and creatinine concentrations.

Whittemore *et al.* (2006) opined that microalbuminuria was always associated with underlying systemic disease.

Dixit *et al.* (2007) detected antigen-specific immune complexes in urine of patients with lymphatic filariasis which provides a noninvasive means of assessing the extent of renal damage in such patients. The renal damage was attributed to the passage of immune complexes through the filtering structures of the kidney into the urine.

Raila *et al.* (2007) stated that urinalysis in a 14-month-old bernese mountain dog with chronic renal failure revealed a specific gravity of 1.020 and an overt proteinuria with the abnormal urine protein creatinine ratio (UPC) of 2.50.

Wehner *et al.* (2008) studied associations between proteinuria, systemic hypertension and glomerular filtration rate in dogs with chronic renal failure and revealed that proteinuria and systemic hypertension were the risk factors in chronic renal failure.

Brunker *et al.* (2009) suggested that increases in urine NAG and GGT indices allow for earlier detection of renal tubular damage in dogs which would enable adjustment of the clinical management of affected dogs to decrease morbidity associated with acute tubular injury and acute tubular necrosis.

Changes in urine pH significantly affected the urine GGT index but not the urine NAG index. Neither index changed significantly with changes in body surface area.

2.9. GROSS AND HISTOPATHOLGY

Otto and Jackson (1969) studied pathology of canine dirofilariasis and stated that changes were seen in lungs, heart and liver. Pulmonary lesions included endarteritis and thickening of the intima of pulmonary artery and the formation of thromboemboli. Cardiac changes were limited to dilatation of the right ventricle. Hepatic lesions consisted of passive congestion, centrilobular necrosis and cavernomatous replacement of the central veins.

Waugh *et al.* (1980) reported chyluric associated glomerulonephritis in patients with lymphatic filariasis. Immunoflourescent and electron microscopic studies showed mesangial deposits of immunoglobulins and complement which suggested that glomerulonephritis in patients with filariasis might be of an immune complex type.

Kamalu (1991) reported gross and histopathological changes in the liver, spleen, heart and lung of dogs massively infected with adults worms of *Dirofilaria repens* with high microfilaraemia in blood and suggested that the toxic and immunological effects of this parasite involved in the pathogenesis of the disease. The lesions were squamous metaplasa of the bronchial epithelium indicating chronic bronchitis, congestion of capillaries and atlectasis in lungs, centrilobular fatty degeneration, congestion, cirrhosis and mononuclear cellular infiltration were observed in liver, congestion, thickening of trabeculae and diffuse fibrosis throughout the pulp in spleen, congestion and haemorrhage in the cortex and medulla, nephrosis, interstitial nephritis and areas of fibrosis in the interstitial tissue in kidney and cardiac lesion included myocarditis. Erythrophagocytosis and haemosiderosis in lungs, liver, spleen, heart and kidney.

Mozos *et al.* (1992) reported an angiocentric and pyogranulomatous dermatitis associated with microfilariae as the most frequently observed histopathological changes.

Paes-de-Almeida *et al.* (2003) suggested that the pathogenesis of kidney disease in dirofilariasis was associated with deposition of immune complexes in the glomerular basement membrane. Thickening of the glomerular basement membrane (GBM), the presence of dense deposits in the GBM, and foot process effacement were the most frequent lesions observed in the affected dogs. The rate of thickening of the glomerular basement membrane always depends on the duration of infection.

Histopathological lesions of *Dirofilaria repens* infected dog included vacuolar ganglyocytic cerebral change and congestion with vasogenic edema in brain, interstitial chronic pancreatitis with proliferative chronic inflammation of the pancreatic ducts, proliferative glomerulonephritis with disseminated glomerular sclerosis, atrophic lymph nodes with depleted lymphocytes , cirrhotic and icteric liver, focal mucosal fibrosis with infiltration of the globule leukocytes in the stomach mucosa, lungs with congestion, alveolar emphysema, thrombosis of the branches of the pulmonary artery, myofibrillar degeneration with hemorrhages in the myocardium, catarrhal chronic inflammation in the small intestine and purulent inflammation in subcutaneous tissue. Examination of the blood smears and tissue imprints of the liver, lymph node and lungs revealed the presence of numerous microfilariae. The authors suggested that this pathogenesis of dirofilariasis was associated with the toxic and immunological effects caused by the parasite in the host body (Dzaja *et al.*, 2008).

2.10. TREATMENT

2.10.1. Ivermectin

Blair *et al.* (1983) reported that single oral dose of ivermectin @ 0.2 mg/kg,0.05mg/kg and 0.0125mg/kg was highly efficacious against the *Dirofilaria immitis* microfilariae in dogs and removed all the microfilariae within 24 hours.

Suderman and Craig (1984) reported that ivermectin @ 200µg/ kg body weight has an efficacy of 99.9% against *Dirofilaria immitis* microfilariae in naturally infected dogs.

Neer and Hoskins (1989) opined that ivermectin eliminates virtually all microfilariae in approximately 90% of dogs within three weeks of administration of a single oral dose of 50 µg/ kg body weight.

Paul *et al.* (1991) reported that adverse reactions with ivermectin have never been reported in collies at dosages less than 100µg/ kg body weight and so collies could safely receive ivermectin at the prophylactic dose.

Calvert and Rawlings (1993) stated that microfilarial counts decrease rapidly during the first 24 hours after a single oral microfilarial dose of ivermectin @ 50µg/ kg body weight and dogs with heavy microfilarial counts may experience acute adverse reactions when ivermectin administrated at high dosages. Ivermectin @ 6-11µg/ kg body weight could be used as an effective once monthly prophylactic drug for non microfilaraemic dogs.

Blagburn (1994) stated that 96% of microfilaraemic dogs were cleared of microfilaria following a single oral dosage of ivermectin @ 50µg/ kg body weight.

Dzimianski (1994) suggested that canine microfilariosis could be effectively prevented by monthly oral dosage of ivermectin 6µg/ kg body weight.

McCall *et al.* (1996) stated that prophylactic dose of ivermectin @ 6 to 12 µg/kg PO monthly in dogs and @ 25 µg/kg PO monthly in cats had a reliable reach-back effect of 2 months.

McCall *et al.* (1998) indicated that ivermectin have partial adulticidal properties when used continuously for 16 months.

Radhika *et al.* (1999) opined that single subcutaneous injection of ivermectin @ 200 and 333 µg/kg body weight were equally effective against *Dirofilaria repens* microfilariae.

Tarello (2000) stated that canine dirofilariasis due to *Dirofilaria repens* could be treated successfully with a single subcutaneous dose of ivermectin @ 50µg/ kg body weight.

Phantana *et al.* (2002) investigated the efficacy of treating cats, naturally infected with *Brugia malayi*, with a single dose of ivermectin (0.05 and 0.1 µg/kg orally and 0.2, 0.4 and 1 mg/kg injected subcutaneously) The results indicated that the microfilarial density of all ivermectin-treated cats significantly dropped after 30 and 60 days of treatment. In cats receiving 0.2 mg/kg, the number of microfilariae decreased by 75–90% on day 30 after therapy and further reduced to nearly 100% on day 60 after receiving a repeat dose given on day 30.

Suprabha and Devada (2003) stated that ivermectin @ 200 µg/ kg body weight subcutaneously once weekly for 2 weeks was effective against microfilariosis caused by *Dirofilaria repens.*

Ghulam *et al.* (2005) opined that canine heartworm disease could be successfully treated using a combination of thiacertarsemide, ivermectin and aspirin.

Soulsby (2005) reported that an oral administration of ivermectin @ 0.05-0.1 mg/kg body weight reduced microfilaremia by 90% within 24 hrs

Bazzocchi *et al.* (2008) stated that treatment with ivermectin (IVM; 6µg/kg per os weekly) combined with doxycycline (DOXY; 10 mg/kg/day orally from Weeks 0–6, 10–12, 16–18, 22–26 and 28–34) resulted in significantly faster reduction of circulating microfilariae and higher adulticidal activity compared with either ivermectin or doxycycline alone.

Chittrakarn *et al.* (2008) reported that the pharmacokinetics of ivermectin in cats receiving a single dose of 0.2 mg/kg by subcutaneous injection revealed a rapid absorption, higher distribution, slow elimination and high possibility for the elimination of *Brugia malayi* microfilariae from currently endemic regions. The serum ivermectin concentrations was measurable only up to 25 days post-treatment, so repeated monthly doses will be required to maintain drug levels.

2.10.2. Levamisole

Bradley (1976) evaluated the microfilaricidal efficacy of levamisole resinate in dogs infected naturally or experimentally with *Dirofilaria immitis* by administering @ 5.5mg/kg or 11mg/kg body weight /day for 10 days and suggested that levamisole resinate when given @ 5.5mg/kg body weight for 10 days was not effective as a microfilaricide and microfilarial counts were reduced in all dogs at this dosage, but never to zero. Levamisole resinate @ 11mg/kg body weight the microfilaria counts were reduced to zero with in 10 days.

Carlisle *et al.* (1984) evaluated the effectiveness of levamisole hydrochloride against the microfilaria of *Dirofilaria immitis* in dogs and opined that levamisole hydrochloride was an efficient microfilaricide at the dose rate of 10 mg/kg twice daily for 14 days. Increasing the dose rate every 3 days to 10 mg/kg twice daily and maintaining it at 10 mg/kg twice daily for a further 8 days, was also effective but a dosage of 5 mg/kg twice daily for 21 days was less effective and a dose rate of 20 mg/kg was effective, but toxic.

Radhika *et al.* (1999) opined that levamisole hydrochloride @7.5mg/kg body weight subcutaneously for seven consecutive days could be used effectively in canine dirofilariasis.

Dillon (2000) reported that administration of levamisole hydrochloride @ 10-12 mg/kg body weight orally for 30 days was found to be effective in canine microfilariosis.

2.10.3. Diethyl Carbamazine

Ottesen *et al.* (1997) reported that the current antifilarial therapies in human beings were restricted to diethyl carbamazine or ivermectin in combination with albendazole.

2.10.4. Tetracycline

Bandi *et al.* (1999) opined that tetracycline was very effective in blocking embryo development in two filarial nematodes *Brugia pahangi* and *Dirofilaria immitis* and also inhibiting the transovarian transmission of their endosymbiont, *Wolbachia*.

2.10.5. Milbemycin Oxime

McCall *et al.* (1996) stated that prophylactic dose of milbemycin oxime @ 0.5 to 0.99 mg/kg PO monthly in dogs and @ 2 mg/kg PO monthly in cats had a reliable reach-back effect of 2 months.

Radhika *et al.* (1999) opined that single oral dose of milbemycin oxime @ 0.5 mg/kg body weight was found to be the most effective drug for microfilariosis.

2.10.6. Selamectin

Thomas (1999) opined that selamectin is a semisynthetic macrolide that is applied topically (6 to 12 mg/kg for dogs and cats) once a month for

prophylaxis. At the preventive dose, it is effective at preventing heartworm infection as well as killing fleas and flea eggs, sarcoptic mange mites, ticks, and ear mites.

Materials and methods

3. MATERIALS AND METHODS

The study was conducted in the Department of Clinical Veterinary Medicine, College of Veterinary and Animal Sciences, Mannuthy during the period of 2007-2009.

3.1. SELECTION OF ANIMALS

Dogs presented to Veterinary College Hospital, Mannuthy and University Veterinary Hospital, Kokkala from different parts of Kerala were utilized for the study. Hundred dogs of both sexes belonging to various breeds and above 6 months of age presented with clinical signs suggestive of microfilariosis like fever, anorexia, vomiting, conjunctivitis, lymphangitis, hair loss, limb and scrotal oedema were screened for microfilaria by wet film examination. Wet film examination revealed that 80% of dogs were positive for microfilaria. Staining of blood smear with giemsa (1:10) demonstrated that 16 out of 80 dogs were positive for sheathed microfilaria and remaining were nonsheathed. Out of these 50 (nonsheathed) microfilaremic dogs were selected and treated at random with five schedules of treatment so that each schedule consisted of ten animals each (Group I, II, III, IV and V) Sheathed microfilaraemic animals were considered as a separate group for treatment trial. All these animals were subjected to periodic wet film examination on 2^{nd}, 4^{th} and 7^{th} day of treatment to assess the treatment response.

Animals of different groups will be treated as follows:

Group I- Single oral administration of ivermectin @50 µg/kg body weight.

Group II- Single oral administration of ivermectin @100 µg/kg body weight.

Group III - Single oral administration of ivermectin @200 µg/kg body weight.

Group IV- Oral administration of levamisole hydrochloride @ 5mg/kg body weight daily for 7 days.

Group V- Oral administration of levamisole hydrochloride @ 10mg/kg body weight daily for 7 days.

Group VI- Oral administration of levamisole hydrochloride @ 10 mg/kg body weight daily for 7 days since initial treatment trial as in the case of group I,II,III and IV were not satisfactory.

3.2. PROCEDURES ADOPTED

3.2.1. Clinical Examination of the Patient

Detailed history and results of clinical examination were recorded as per the proforma (Annexure I). Clinical examination of the patients were conducted as per the protocol suggested by Houston (2000).

3.2.2. Parasitological Investigations

3.2.2.1. Staining Technique

About 3 ml of blood was drawn from the cephalic vein and allowed to clot. The serum obtained is centrifuged at 3000 rpm for 5 minutes. The sediment was examined for microfilariae. Thin smears were prepared from sediment and air dried.

3.2.2.1.1. Giemsa Staining

The smears were fixed in absolute alcohol for one minute and stained with Giemsa stain (1:10) and examined under oil immersion objective of the microscope. Identification of microfilariae was done based on the morphological peculiarities.

3.2.2.1.2. Histochemical Staining

Smears were also fixed in chilled absolute acetone for one minute and kept at -20ºC for histochemical staining for the detection of acid phosphatase activity as reported by Chalifoux and Hunt (1971). The smears fixed in chilled absolute acetone were stained with Acid Phosphatase Leukocyte Kit (Far Diagnostics, Italy) as per manufacture's procedure. This kit utilizes Naphthol AS-BI phosphatase as the substrate and pararosanilin as the chromogen. Identification/speciation of microfilaria was done according to Chalifoux and Hunt (1971) and Kobasa *et al.* (2004).

3.2.2.2. Micrometry

The measurements of microfilariae were taken using stage and optical micrometry and identified the species by comparing with standard measurements of the parasites as suggested by Soulsby (2005).

3.2.2.3. Immunological Test

Sera from 16 dogs with sheathed microfilariae, two dogs with non sheathed microfilariae and two healthy controls were utilized for immunological test using Signal MF Reagent (Filarial Antibody detection spot/immunodot test kit (Span diagnostics, Gujarat)) developed by Baskar *et al* (2004) under standard conditions for the detection of antibodies as per manufacture's procedure.

3.2.2.4. Polymerase Chain Reaction (PCR) and Sequencing of Amplicon

3.2.2.4.1. Extraction of Filarial DNA from Blood Sample

Five hundred microliter of whole blood were lysed in 500 µl lysis buffer (50 mM Tris, pH 8.0; 100 mM EDTA,100 mM Nacl, 1% SDS) for one hour at 55°C. The samples were then digested with 100 µl proteinase K (10 mg/ml) for 16 hr at 55°C. The DNA was extracted with standard phenol/chloroform method of Sambrook and Russel (2001), precipitated by ethanol and then resuspended in

30 µl of TE buffer (10mM Tris - HCl (pH 8.0), 1 mM EDTA). The enzyme activity was inactivated by incubation at 90°C for10 minutes. The DNA obtained were stored at –20°C for later use.

3.2.2.4.2. Primers Selected

Primers that are specific for trans-spliced leader exon 1 (SLX1 and SLX2) region of *Brugia* species (Chansiri *et al.*, 2002) and pan-filarial primers (DIDR-F1 and DIDR- R1) to identify and discriminate six species of microfilariae known to infect dogs by amplifying ribosomal DNA spacer sequences (Rishniw *et al.*, 2006) were used in this study. All the primers were developed at Integrated DNA Technologies (IDT), USA.

The Primers were

Trans-spliced leader exon 1

SL X1 (forward) SLX1- GTCTACGACCATACCACGTTGA

SLX2 (Reverse) SLX2 - GAAACATTCAATTACCTCAAAC

Pan-filarial primers

DIDR-F1 – AGT GCG AAT TGC AGA CGC ATT GAG

DIDR-R1 - AGC GGG TAA TCA CGA CTG AGT TGA

3.2.2.4.3. PCR Amplification and Sequence Analysis

Twenty five microliter of PCR reaction mixture was carried out by using 0.1 mM of each primer (forward and reverse) in the presence of 1x PCR buffer, 0.1 mM dNTP, 1.5 mM Mgcl2 and 1 unit of *Taq* polymerase. The PCR mixtures were heated at 95°C for 4 min prior to monitoring the PCR cycle. One cycle of PCR was consisted of denaturation at 95°C for 60s, annealing at 59°C for 60s and polymerization at 72°C for 90s.The cycle was proceeded for additional 29 cycles.

Ten microlitre of PCR products of approximately 294 bp in size was analysed by using 1.5% agarose gel electrophoresis, stained by ethidium bromide and visualized under ultraviolet transluminator. The PCR product obtained was sequenced at Genie, Bangalore and the sequences were then analysed using Basic Local alignment Search Tool (BLAST).

3.2.3. Clinical Investigations

3.2.3.1. Electrocardiography

Electrocardiogram of the patients were recorded using BPL CARDIART-6108® ECG machine. Three standard bipolar limb leads (I, II, III) and three augmented unipolar limb leads (aVR, aVL, aVF) were used for the study.

Animals were placed in right lateral recumbancy and the electrodes were placed over the elbow and stifle joints of the forelimbs and hind limbs respectively after applying jelly (Goodwin, 2001 and Martin, 2002). ECG was recorded at a paper speed of 25mm per second and sensitivity of 1mv=10mm and were evaluated.

3.2.3.2. Ultrasonography

Ultrasonography was done using Mindray DC-6 Vet ultrasound machine with a sector transducer of frequency ranging from 3.5-7.5 MHz. Ultrasonographic evaluation of liver and kidney of affected animals were done on the day of presentation to evaluate the hepatic and renal involvement of the condition.

3.2.3.3. Radiography

Radiographs of the limbs of animals with unilateral limb oedema were carried out to rule out orthopaedic abnormalities.

3.2.4. Clinical Pathology

3.2.4.1. Sample Collection and Analysis

Blood samples were collected on the day of admission and on 7th day from the cephalic vein of each dog under sterile technique between 9 am to 4 pm. About 10 ml of urine was collected from selected animal on the day of admission for urinalysis.

3.2.4.2. Haematology

Blood samples were collected from 66 microfilaraemic dogs and six nonmicrofilaraemic healthy controls selected for detailed study. About 2 ml of blood was collected in a clear, dry, test tube with EDTA dipotassium salt @ 1 mg/ml of blood as anticoagulant for erythrogram, leucogram and platelet counts using standard technique as described by Feldman *et al*. (2000). The values of haemogram of microfilaraemic dogs were compared with healthy controls.

3.2.4.3. Serum Biochemistry

About 5 ml of blood was collected from 66 microfilaraemic dogs and six nonmicrofilaraemic healthy controls, in a clean dry test tube without anticoagulant, allowed to clot and centrifuged at 3000 rpm for 15 minutes. Sera thus separated were stored at -20°C until further analysis. Serum samples were colorimetrically analyzed for the activities of alanine amino transferase (ALT), alkaline phosphatase (ALP), aspartate amino transferase (AST), blood urea nitrogen and creatinine, total proteins, albumin, globulins and A:G ratio. All the biochemical analysis were performed in Secomam Basic semi autoanalyser as per the manufacturer instructions (Kits from M/S Agappe diagnostics). The values obtained for microfilaraemic dogs were compared with healthy controls.

3.2.4.4. Urinalysis

Urine samples collected from selected microfilaraemic dogs and six nonmicrofilaraemic healthy controls on the day of admission were utilized for analysis of specific gravity , bile pigments and protein (Uristick method), Urine protein creatinine ratio (UPC), γ glutamyl transferase (γ GT) and ALP using standard kits (Merck, Mumbai).N-Acetyl-β-D-Glucosaminidase (NAG) was estimated as per the manufacturer's instructions (Kits from Far Diagnostics, Italy).

3.2.5. Autopsy – Gross and Histopathology

Two animals died during the course of treatment were subjected to post mortem examination. Representative tissue samples of heart, lungs, liver and kidney were fixed in 10% formalin and were processed and embedded as described by Sheehan and Hrapchak (1980).The sections were stained using Haematoxylin and Eosin, as per the staining technique followed by Bancroft and Cook (1995).

3.2.6. Statistical Analysis

The data obtained in this study were computed and statistically analyzed using paired 't' test according to Snedecor and Cochran (1980).

Results

4. RESULTS

4.1. SCREENING OF DOGS FOR MICROFILARIAE

Hundred dogs of both sexes belonging to various breeds and above 6 months of age presented to Veterinary College Hospital, Mannuthy and University Veterinary Hospital, Kokkala from different parts of Kerala with clinical signs suggestive of microfilariosis like fever, anorexia, vomiting, conjunctivitis, hair loss, lymphangitis, limb and scrotal oedema (Plate 1a and b) were screened for microfilaria by wet film examination. Wet film examination revealed circulating microfilaria in 80% of dogs. Staining of blood smear with giemsa (1:10) demonstrated that 16 out of 80 dogs were positive for sheathed microfilaria and remaining were nonsheathed (Fig. 1).

4.2. EPIDEMIOLOGY

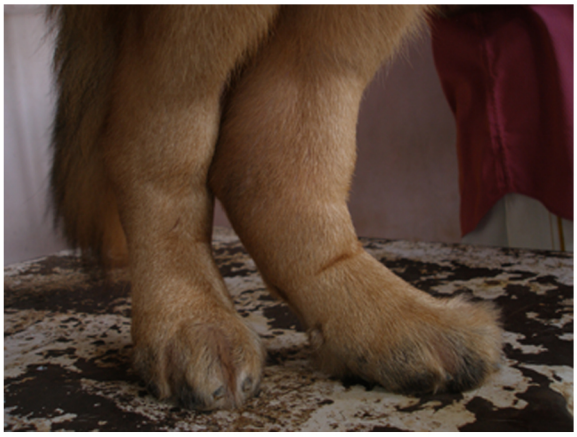
4.2.1. Age

About 64.06 percent of dogs with nonsheathed (NS) microfilariae were in 2 to 4 years of age group followed by dogs older than 4 years (28.13%) and dogs below 2 years (7.81%). The mean age of dogs with sheathed microfilariae (S) were 2 to 4 years (Table 1 and Fig. 2).

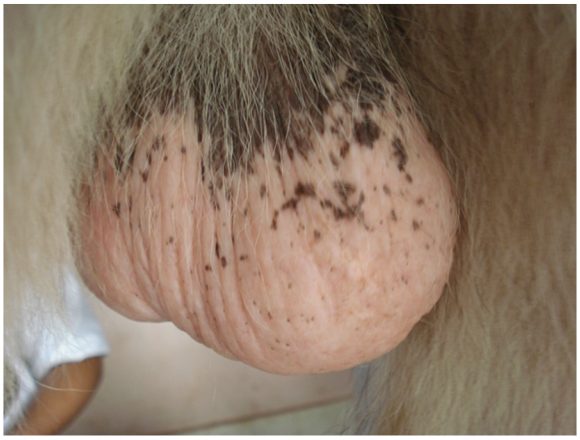
Table 1. Age wise distribution of microfilariosis in dogs

Age (years)	Nonsheathed microfilariae (64)		Sheathed microfilariae (16)	
	Number	Per cent	Number	Per cent
<2	5	7.81	0	0
2 - 4	41	64.06	16	100
>4	18	28.13	0	0

Plate 1a: Clinical signs



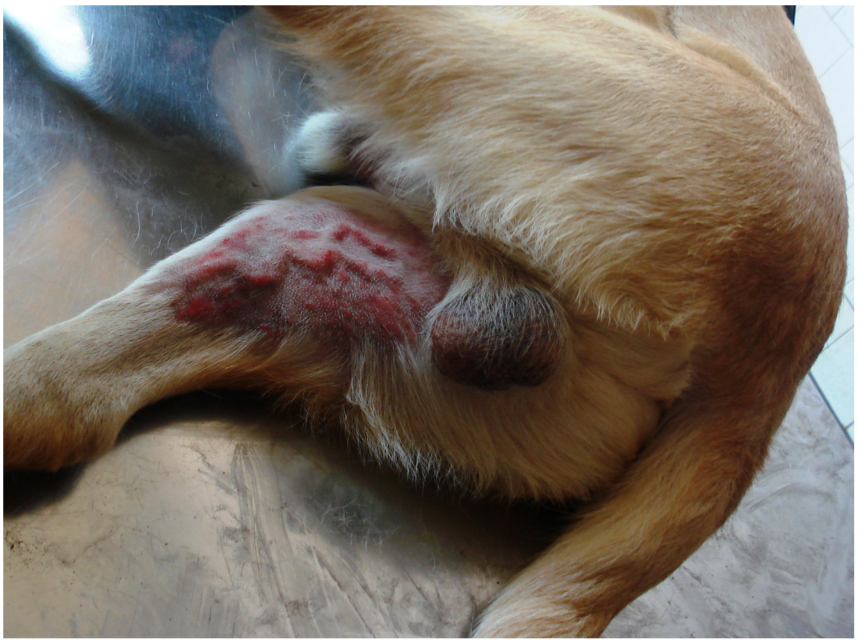
A. Limb oedema



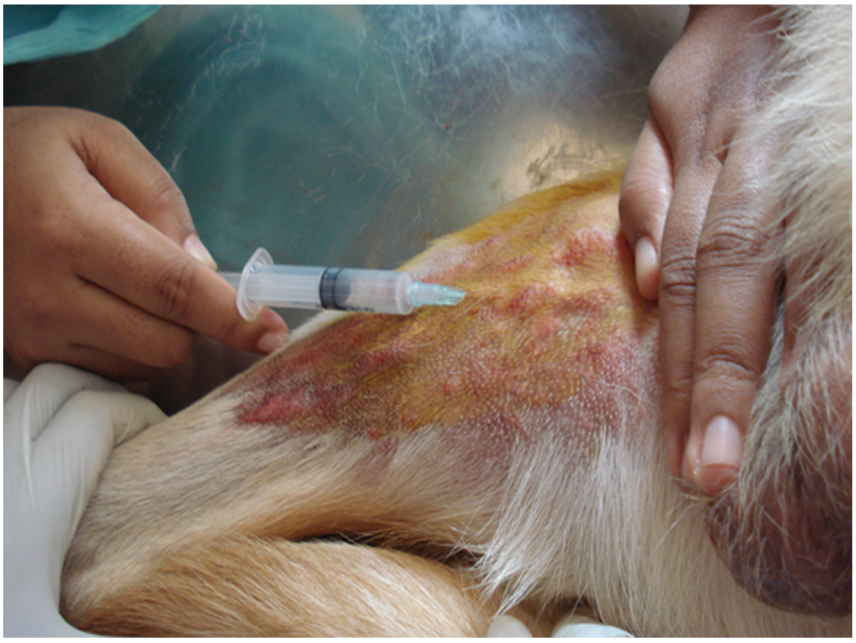
B. Scrotal oedema

C. Conjunctivitis

Plate 1b: Clinical signs



A. Lymphangitis



B. Collection of lymph from dilated lymphatics

Fig.1: Screening of dogs for microfilariosis

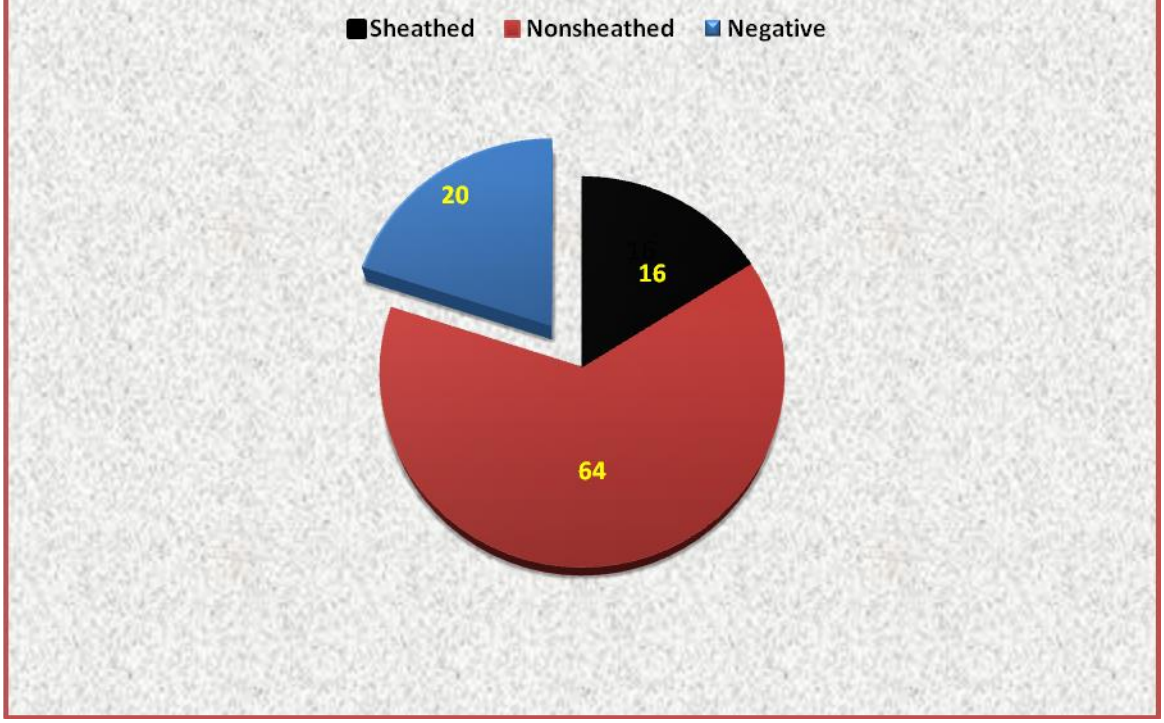
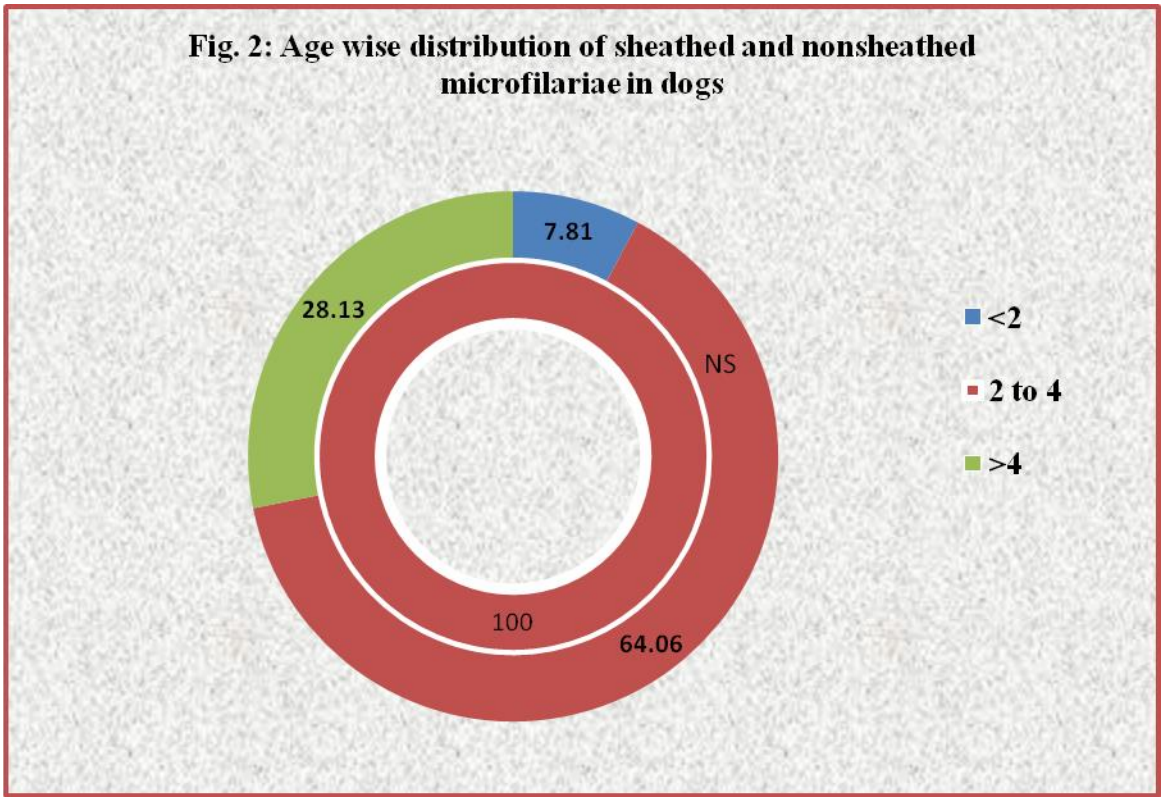


Fig. 2: Age wise distribution of sheathed and nonsheathed microfilariae in dogs



4.2.2. Breed

Out of 64 dogs with nonsheathed microfilariae, the incidence of microfilariosis was more in German Shephered (26.56%) followed by Labrador (23.44%), Dachshund (17.19%), Rottweiler (10.94%), Non-descript (6.25%), Doberman, Boxer, Dalmatian, Spitz, Cocker Spaniel and Great Dane. Seventy five percent of dogs with sheathed microfilariae were belong to Labrador breeds followed by German shephered, Rottweiler, Doberman and Basset hound (Table 2 and Fig. 3 a and b).

Table 2. Breed wise distribution of microfilariosis in dogs

Breeds	Nonsheathed microfilariae (64)		Sheathed microfilariae (16)	
	Number	Per cent	Number	Per cent
German Shephered	17	26.56	1	6.25
Labrador	15	23.44	12	75
Dachshund	11	17.19	0	0
Rottweiler	7	10.94	1	6.25
Non-descript	4	6.25	0	0
Dobermann	2	3.13	1	6.25
Boxer	2	3.13	0	0
Dalmatian	2	3.13	0	0
Spitz	2	3.13	0	0
Cocker Spaniel	1	1.56	0	0
Great Dane	1	1.56	0	0
Basset hound	0	0	1	6.25

Fig. 3a: Breed wise distribution of nonsheathed microfilariae in dogs

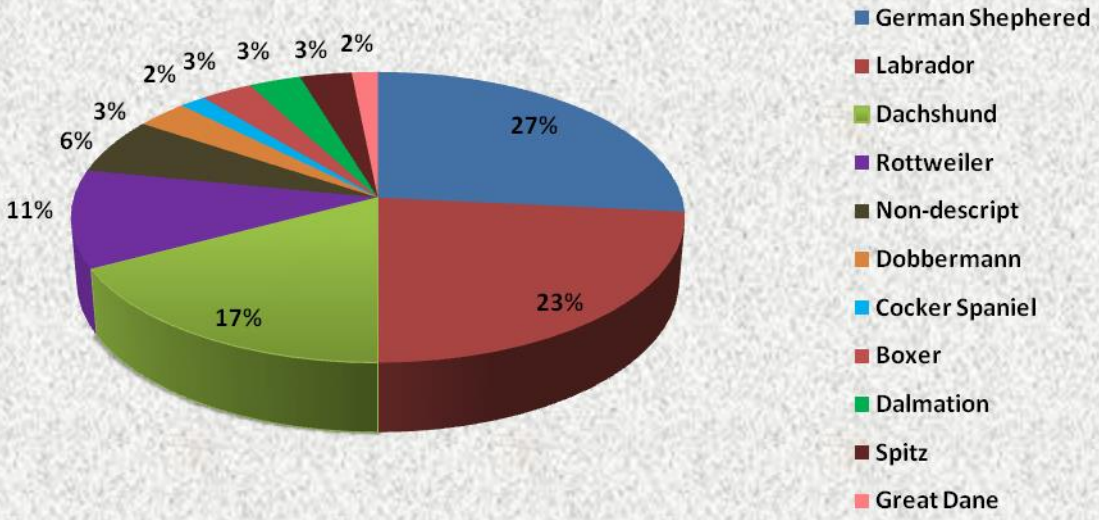
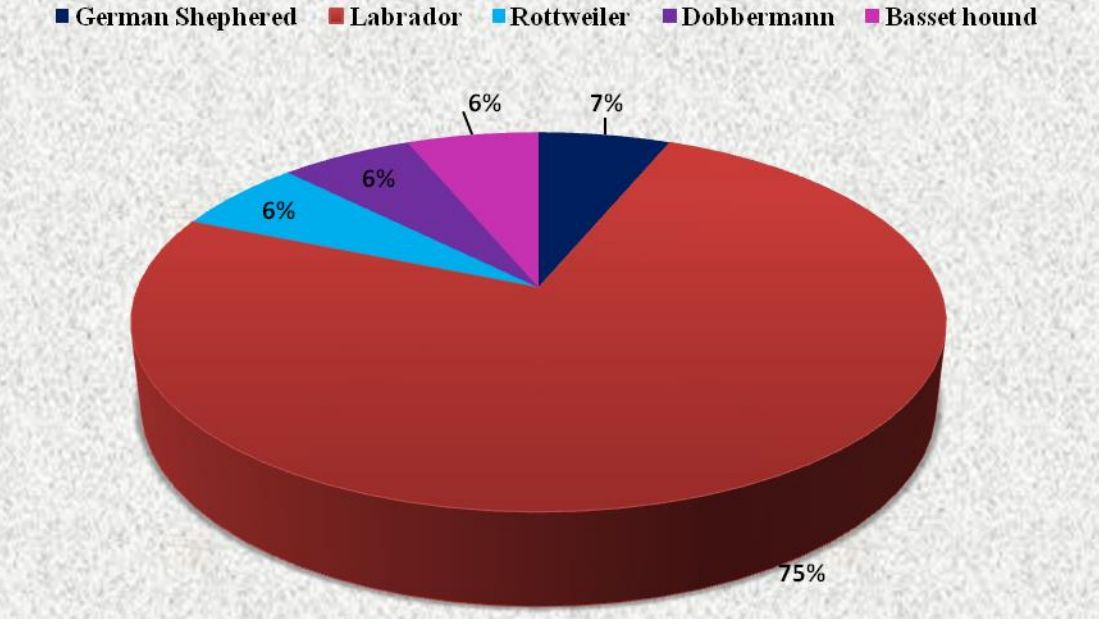


Fig. 3b: Breed wise distribution of sheathed microfilariae in dogs



4.2.3. Sex

Incidence of nonsheathed and sheathed microfilariae in male dogs were 54.69 and 62.5 % respectively and the corresponding values for female dogs were 45.31 and 37.50 % respectively. High infestation rates were recorded in male dogs than females irrespective of the type of microfilariae (Table 3 and Fig. 4).

Table 3. Sex wise distribution of microfilariosis in dogs

Sex	Nonsheathed microfilariae (64)		Sheathed microfilariae (16)	
	Number	Per cent	Number	Per cent
Male dogs	35	54.69	10	62.5
Female dogs	29	45.31	6	37.5

4.3. CLINICAL SIGNS

Major clinical signs common to dogs infected with sheathed and nonsheathed microfilariae were anorexia, fever, vomiting, limb oedema, scrotal oedema, conjunctivitis and hair loss. Limb oedema (31.25%) and conjunctivitis (43.75%) were more in dogs infected with sheathed microfilariae than nonsheathed ones. Lymphangitis was characteristic to dogs infected with sheathed microfilariae, where animal exhibit pain and limping of the affected limb. Respiratory and cardiac signs like cough, dyspnoea and exercise intolerance were more in dogs infected with nonsheathed microfilariae. Other symptoms associated with nonsheathed microfilariae in dogs were corneal opacity (4.69%), epilepsy (3.13%), haemoglobinuria (6.25%) and ascites (4.69%) (Table 4 and Fig. 5 a and b). Nodular lesions on skin of face, neck, base of ear pinnae and tail with adult worms were noticed in one of the animals. The clinical data of the microfilaraemic dogs revealed an average temperature, pulse and respiration of

103ºF, 117/min and 28/min respectively. The mucous membranes were pale to pale roseate.

Table 4. Major clinical signs of microfilaraemic dogs

Clinical signs	Nonsheathed microfilariae (64)		Sheathed microfilariae (16)	
	Number	Per cent	Number	Per cent
Anorexia	60	93.75	14	87.5
Fever	58	90.63	15	93.8
Vomiting	25	39.06	4	25
Cough	5	7.81	0	0
Dyspnoea	8	12.5	0	0
Lymphangitis	0	0	5	31.25
Exercise intolerance	3	4.69	0	0
Limb oedema	14	21.88	5	31.25
Scrotal oedema	8	12.5	2	12.5
Conjunctivitis	3	4.69	7	43.75
Ocular discharge	5	78.13	0	0
Corneal opacity	3	4.69	0	0
Skin lesion/Hair loss	19	29.69	6	37.5
Epilepsy	2	3.13	0	0
Haemoglobinuria	4	6.25	0	0
Ascites	3	4.69	0	0

Fig. 4: Sex wise distribution of sheathed and nonsheathed microfilaria in dogs

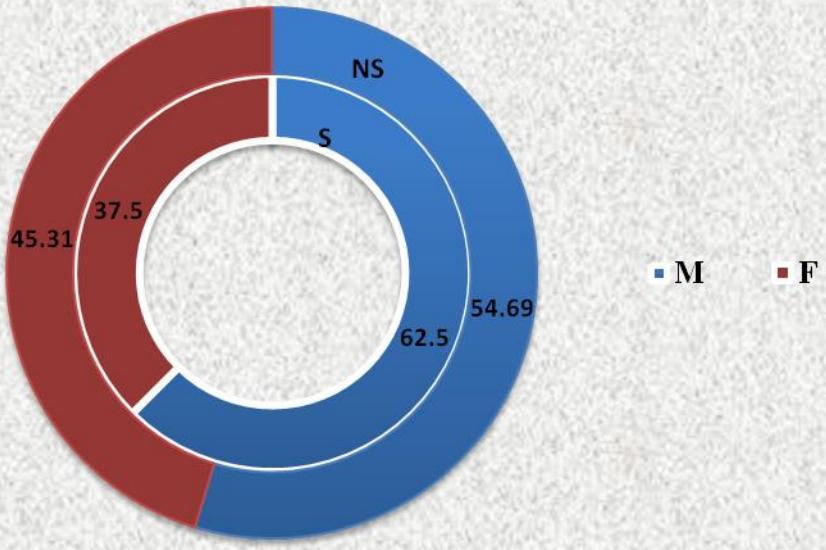
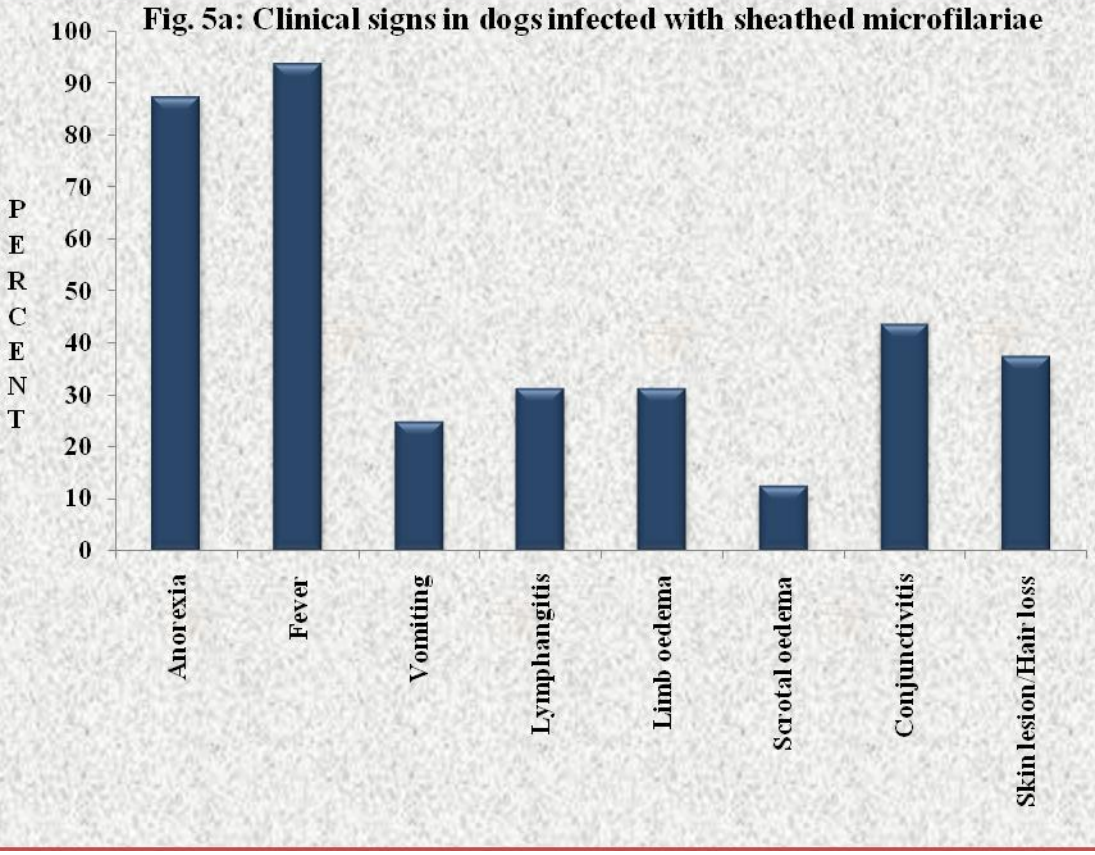


Fig. 5a: Clinical signs in dogs infected with sheathed microfilariae



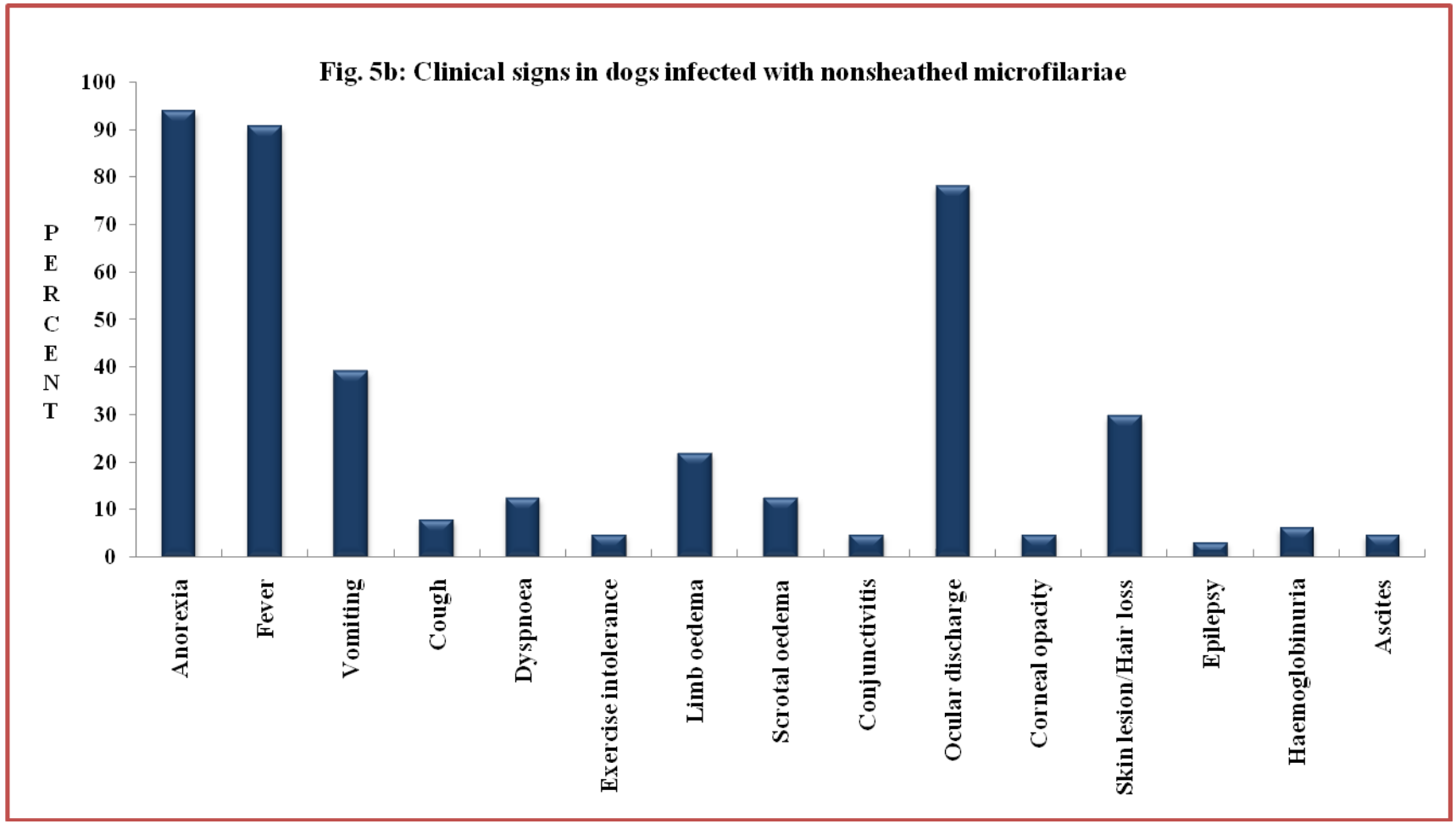


Fig. 5b: Clinical signs in dogs infected with nonsheathed microfilariae
PERCENT
100
90
80
70
60
50
40
30
20
10
0
Anorexia
Fever
Vomiting
Cough
Dyspnoea
Exercise intolerance
Limb oedema
Scrotal oedema
Conjunctivitis
Ocular discharge
Corneal opacity
Skin lesion/Hair loss
Epilepsy
Haemoglobinuria
Ascites

4.4. DIAGNOSIS

4.4.1. Parasitological Investigations

4.4.1.1. Wet Film

Screening of dogs for microfilariosis by wet film examination revealed that 80 out of 100 dogs were positive for microfilariae. In wet film, the microfilariae exhibited four distinct patterns of motility. It appeared snake like with wriggling movement, wriggling cum progressively forward, rapidly forward and sluggish forward movement across the microscopic field. Lymphatic fluid collected from dog with lymphangitis was positive for microfilaria (plate 1b).

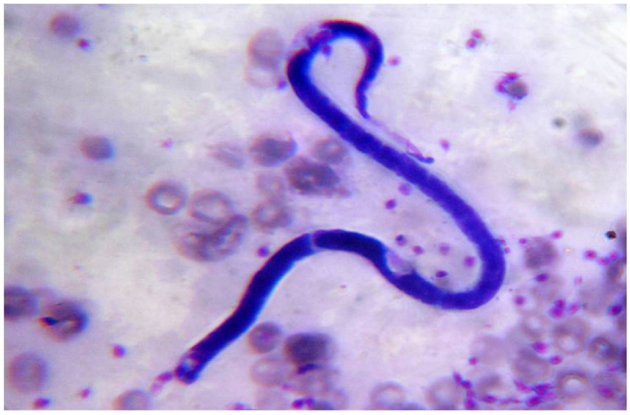
4.4.1.2. Giemsa staining

All the samples found to be positive for microfilariae on wet blood film were positive for Giemsa staining also. Giemsa stained smears of microfilaraemic dogs revealed both sheathed (20%) and nonsheathed microfilariae (80%).

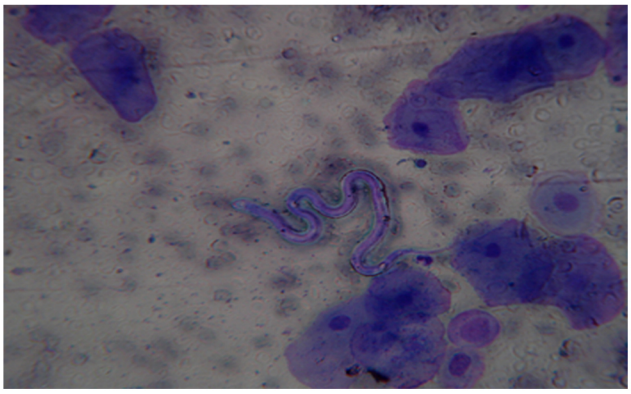
Nonsheathed microfilariae with two distinct morphological peculiarities were observed in stained smears. The microfilariae with a blunt head and a long tapering tail was identified in 60 cases. In the nuclear column, cells did not extend up to the tail tip. Nerve ring, excretory pore and anal pore region were well appreciated in stained smears. In four cases, the microfilariae were smaller with a cephalic hook and button hook like curved tail. Nonsheathed microfilariae with a blunt head and a long tapering tail could be detected on staining the vaginal discharge for exfoliative vaginal cytology and nipple secretion for cytological examination of mammary tumour of two microfilaraemic dogs (Plate 2).

Sheathed microfilariae with pink stained sheath and two discrete overlapping nuclei at the tail end and the length of cephalic space about twice the width was observed in 16 cases. Sheathed microfilariae were smaller in size with

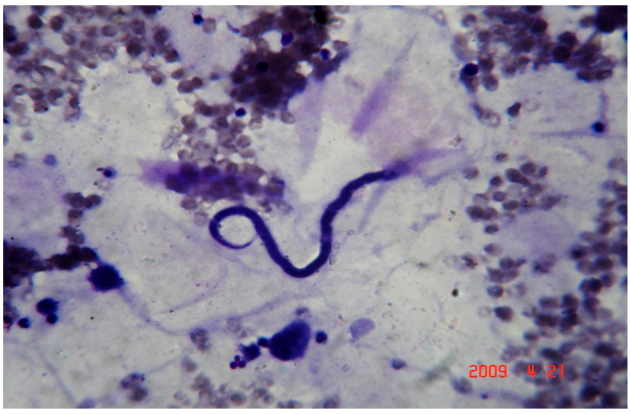
Plate 2: Giemsa staining - Nonsheathed microfilariae



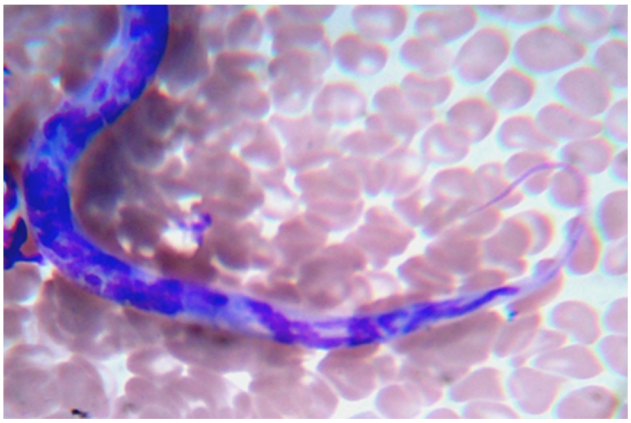
A. Blood smear-
Dirofilaria repens



B. Vaginal smear-
Dirofilaria repens

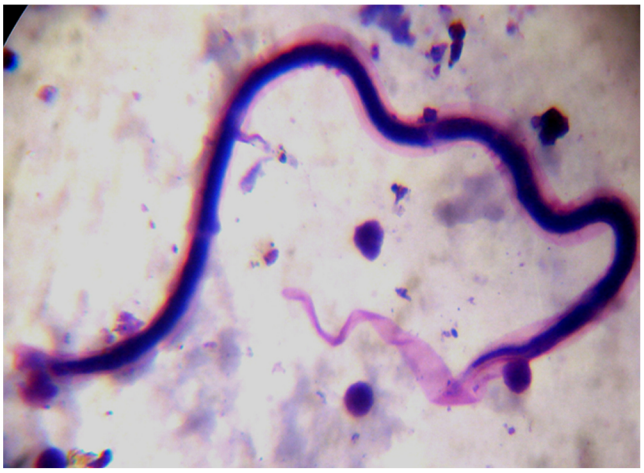


C. Milk smear-
Dirofilaria repens

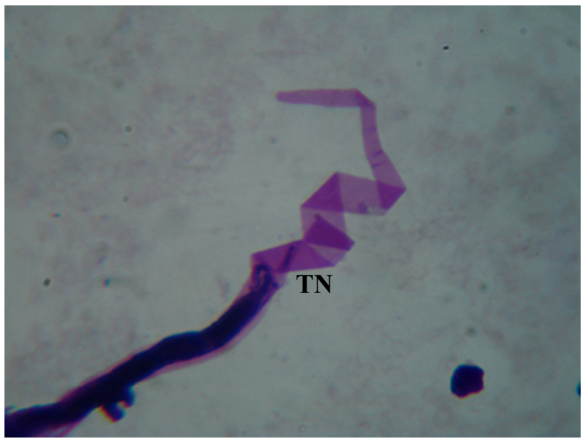


D. Tail end -
D. reconditum

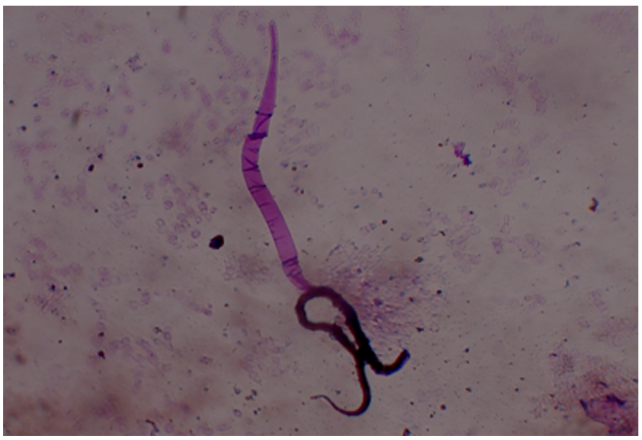
Plate 3: Giemsa staining - Sheathed microfilariae



A. *Brugia malayi* (100X)



B. *Brugia malayi* - Terminal nuclei (TN) at tail end (100X)



C. *Brugia malayi* unsheathing (40X)

secondary kinks and the nuclei extended up to the tip of the tail and the sheath extends beyond the tail tip (Plate 3).

4.4.1.3. Histochemical Staining

Histochemical staining of nonsheathed microfilaria revealed three distinct types: a) microfilaria in which the enzyme activity demonstrated at the anal pore (AN) only b) microfilaria in which the enzyme activity demonstrated at the anal pore (AN) and central body region (CB) (diffuse) c) microfilaria exhibiting uniform enzyme activity, but less intense activity cranial to the excretory pore. The first two patterns were seen in sixty cases and the third in four cases (Pate 4).

Histochemical staining of sheathed microfilaria also revealed three distinct types: 1) microfilaria in which enzyme activity restricted to amphids, excretory and anal vesicles and phasmids 2) microfilaria in which enzyme activity restricted to excretory and anal vesicles 3) microfilaria exhibiting intense enzyme activity uniformly throughout the body of the organism (plate 5). The first two patterns were observed in 15 cases and the third pattern was seen in a dog imported from Russia.

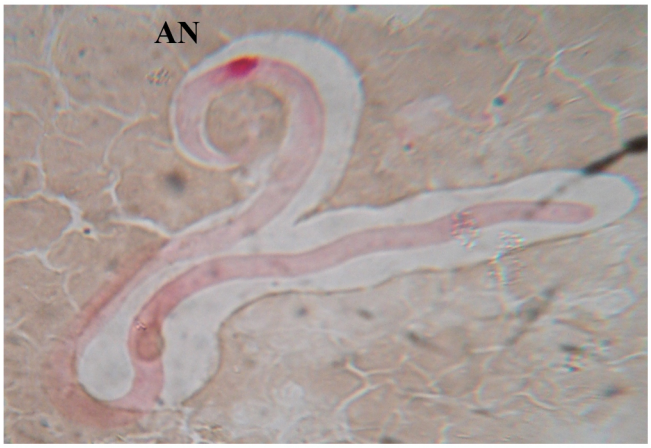
4.4.1.4. Micrometry

The measurements of microfilariae from giemsa/histochemical stained smears were taken using stage and optical micrometry. The mean length and width of nonsheathed microfilariae of type ‘a’ and ‘b’ were 292 ±8.4 and 6 µm respectively and type ‘c’ microfilariae were 265±5.6 and 5 µm respectively. The mean length of sheathed microfilariae of type ‘1’ and ‘2’ were 215±20 and type 3 microfilariae were 280 µm respectively.

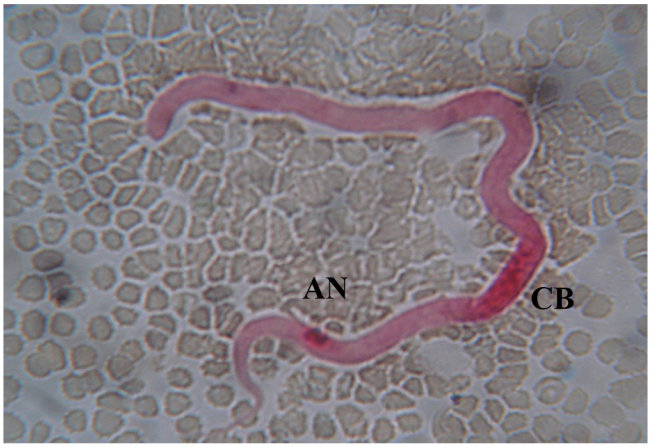
4.4.1.5. Immunological Test

A total of 20 samples were subjected to immunospot test which specifically detects antibodies against brugian and bancroftian filariasis. Out of 16 cases with sheathed microfilariae subjected to immunospot test, 8 samples

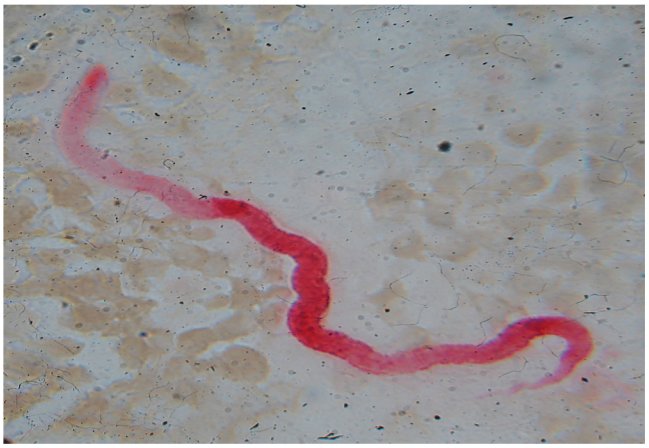
Plate 4: Histochemical staining - Nonsheathed microfilariae



A. *Dirofilaria repens* (Type a)



B. *Dirofilaria repens* (Type b)



C. *Dipetalonema reconditum* (Type c)

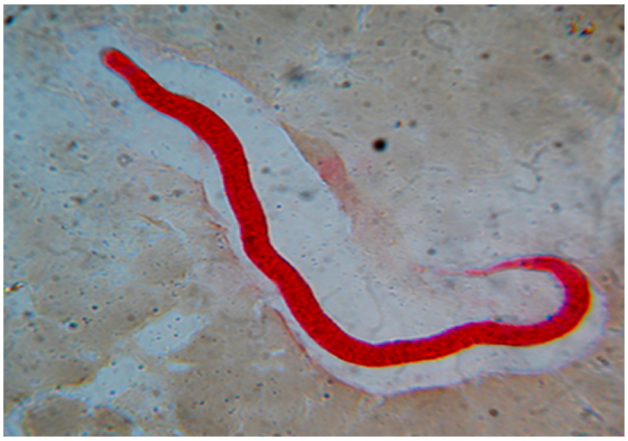
Plate 5: Histochemical staining - Sheathed microfilariae

A. *Brugia malayi* (Type 1)

B. *Brugia malayi* (Type 1)

C. *Brugia malayi* (Type 2)

D. *Brugia pahangi* (Type 3)



showed clear positive reaction, 4 samples showed faint reaction and the other 4 samples showed negative result. Four cases, two each of nonsheathed microfilariae and control animals exhibited negative reaction (Plate 6).

4.4.1.6. Polymerase Chain Reaction and Sequencing of Amplicon

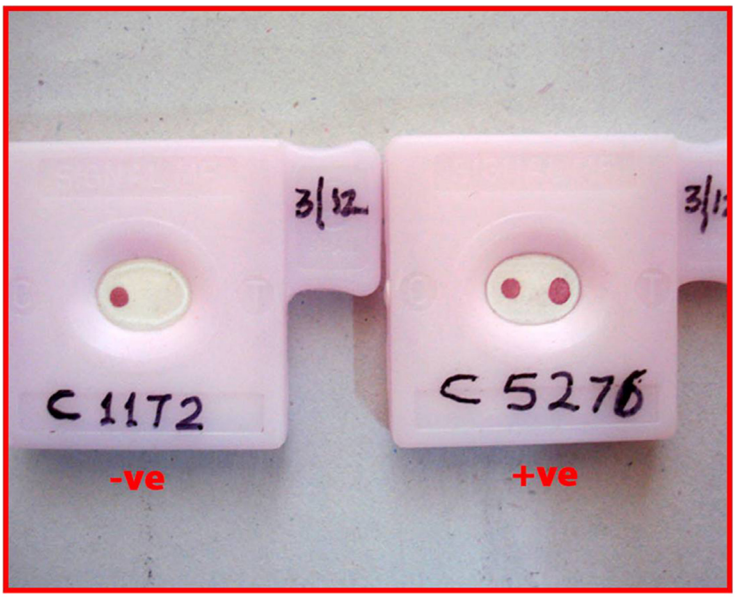
Representative samples were subjected to PCR analysis. Results of PCR analysis using universal primer (DIDR-F1 and DIDR-R1) revealed two bands of 484 bp and 615 bp in case of mixed infection with nonsheathed and sheathed microfilariae. Polymerase chain reaction analysis using *Brugia* specific (SLX 1 and SLX 2) primers revealed a band of 294 bp amplified fragment which corresponds to the SLX gene of *Brugia malayi* microfilaria (Plate 7).

Sequencing of 294 bp PCR product revealed that the amplified product was from a region of 5S rRNA gene and Spliced leader sequence SL1. The sequence obtained when analysed using BLAST revealed 93% homology with a query coverage of 32% with the published *Brugia malayi* gene sequence (D87037.1) and was subsequently submitted to the Genbank data base at the National Centre for Biotechnology Information and assigned accession number FJ717408 (Plate 8).

On sequencing of 484 bp amplicon, 5 .8S rRNA partial sequence and internal transcribed spacer 2 (ITS-2) region was obtained and when analysed using BLAST revealed 94% identity with a query coverage of 62% with the already published partial sequence of 5 .8S rRNA gene of *Dirofilaria repens* (AY693808.1) and was subsequently submitted to the Genbank data base at the National Centre for Biotechnology Information and assigned accession number FJ717410 (Plate 9).

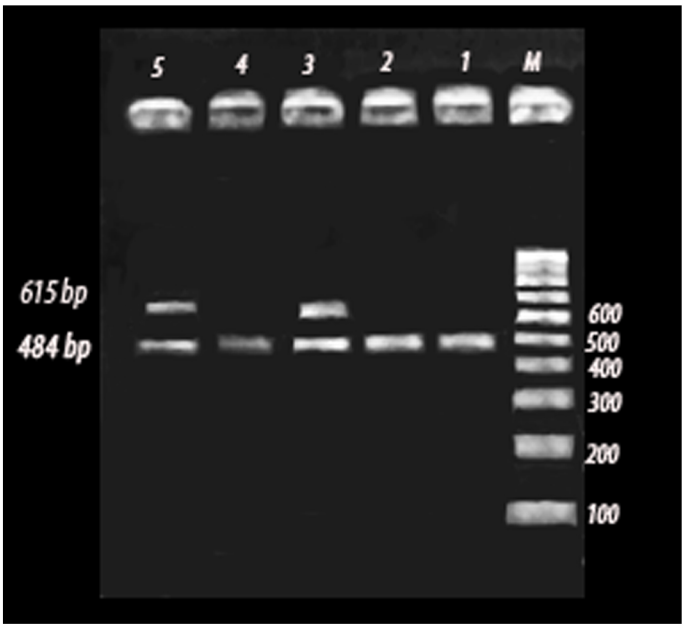
Plate 6 : Immunospot test

A.Immunospot test kit

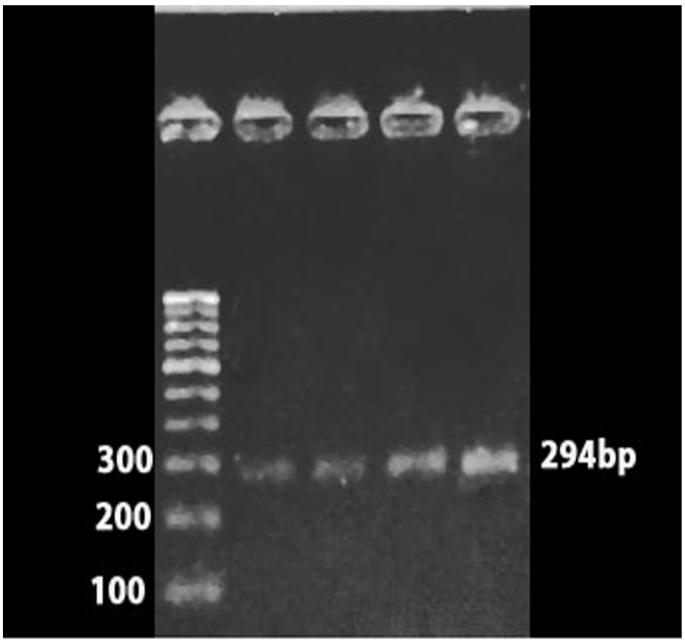


B. Test result

Plate 7: Polymerase Chain Reaction (PCR)



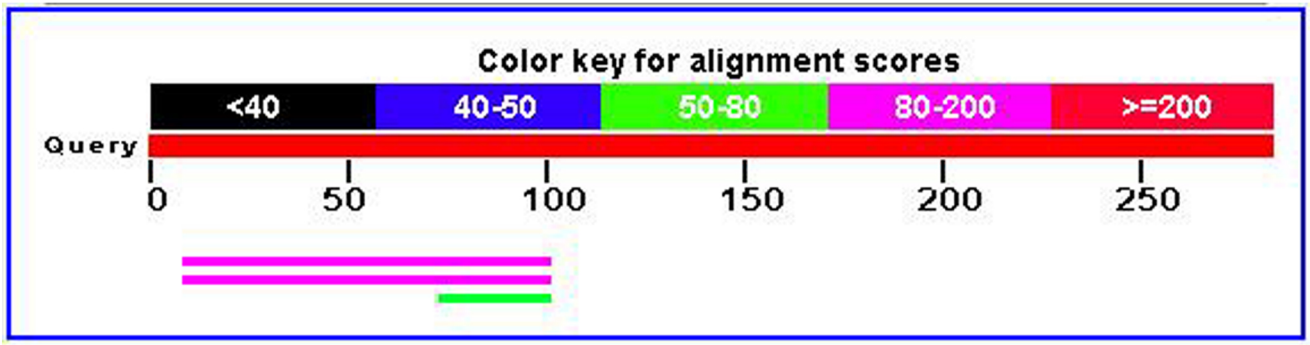
A. Gel electrophoresis of filarial PCR products using pan filarial ITS-2 region primers. Lane M - ladder, Lane 1 to 5 - Sample. 484 bp -*Dirofilaria repens*, 615 bb -*Brugia malayi*



B. Gel electrophoresis of filarial PCR products using SLX primers. Lane M - ladder, Lane 1 to 4 - Sample. 294 bp - *Brugia malayi*

Plate 8: Blast result of 294 bp amplicon

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
D87037.1	Brugia malayi 5S rRNA gene and spliced leader sequence SL1	135	135	32%	7e-31	93%	
AF499131.1	Brugia timori 5S ribosomal RNA gene, partial sequence	126	126	32%	4e-28	91%	
X14846.1	Brugia malayi gene for 5S ribosomal RNA	52.8	52.8	9%	7e-06	100%	



>dbj|D87037.1| Brugia malayi 5S rRNA gene and spliced leader sequence SL1
Length=342

Score = 135 bits (73), Expect = 7e-31
Identities = 86/92 (93%), Gaps = 1/92 (1%)
Strand=Plus/Minus

```
Query  10   GTAGTTAATCCGTCGATCACACCTGCATCATTATACATATAGTTGTTGCGCATTCAACAT  69
            ||| |||| |||||||||| |||||||||||||| |||||||||||| | ||||||||||
Sbjct  265  GTAATTAAACCGTCGATCATACCTGCATCATTAT-CATATAGTTGTTGTGTATTCAACAT  207

Query  70   TCAGCTGATTATTTTTCGGCAAACAGATCAAA  101
            ||||||||||||||||||||||||||||||||
Sbjct  206  TCAGCTGATTATTTTTCGGCAAACAGATCAAA  175
```

Plate 9: Blast result of 484 bp amplicon

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
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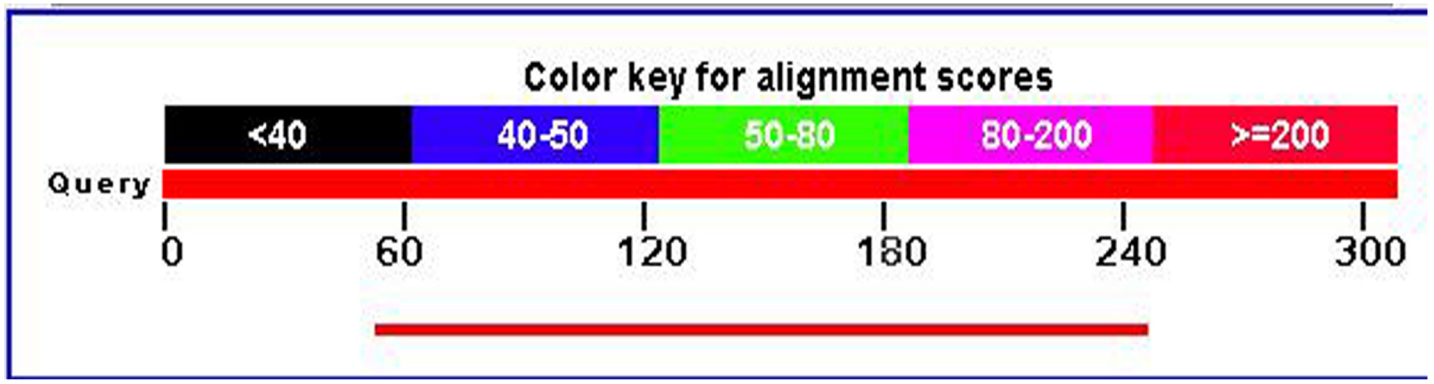

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4.5. CLINICAL INVESTIGATIONS

4.5.1. Electrocardiography

The mean ECG values (lead II) of microfilaraemic dogs were presented in Table 5. P duration, P amplitude, P-R interval, R amplitude, QRS duration, ST segment, T amplitude and Q deepening were 0.07 ± 0.11 sec, 0.27 ± 0.03 mV, 0.11 ± 0.02sec, 1.13 ± 0.24mV, 0.05 ± 0.01sec, 0.23 ± 0.02 mV, 0.27 ± 0.04mV and 0.28 ± 0.05 mV respectively. Abnormalities noticed were increased P duration, Q wave deepening and ST coving. Notching of P wave and peaking of T wave were noticed in two of the cases (Plate 10).

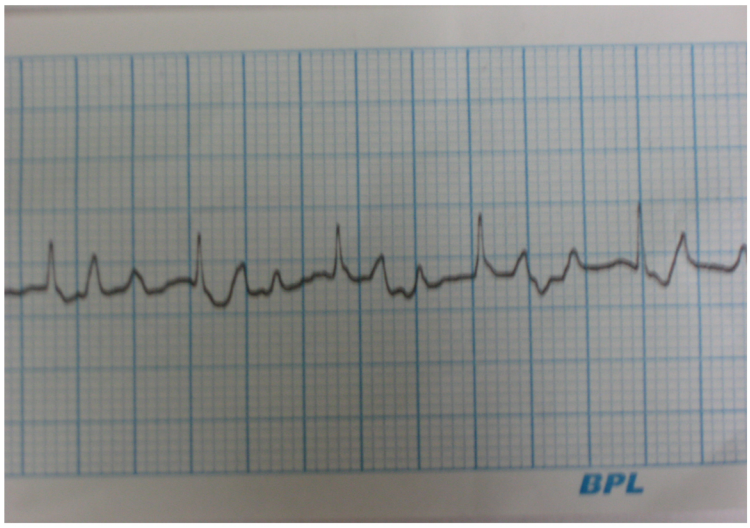
Table 5: Electrocardiographic values (mean±SE) of microfilaraemic dogs

Parameters	Lead II ECG values	Normal ECG values
P duration (sec)	0.07 ± 0.11	0.04
P amplitude (mV)	0.27 ± 0.03	0.4
P-R interval (sec)	0.11 ± 0.02	0.06 – 0.13
R amplitude (mV)	1.13 ± 0.24	0.9 – 2.8
QRS duration (sec)	0.05 ± 0.01	0.05
ST segment (mV)	0.23 ± 0.02	0.1
T amplitude (mV)	0.27 ± 0.04	$\leq 1/4^{th}$ R amplitude
Q deepening (mV)	0.28 ± 0.05	0.5

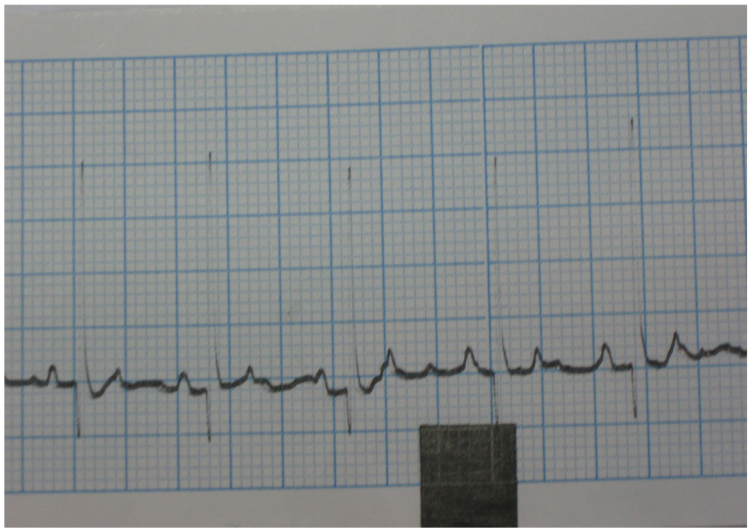
4.5.2. Ultrasonography

Major changes observed in ultrasonogram of liver were focal hyperechoic areas to increased echogenicity of hepatic parenchyma and dilated hepatic vessels. Ultrasonographic appearance of kidneys revealed hyperechoic areas in medullary region and renal pelvis. The echogenicity of cortex was increased with decreased cortical thickness and corresponding reduction in corticomedullary delineation (Plate 11).

Plate 10: Electrocardiogram



A. Increased P duration



B. Q wave deepening

4.5.3. Radiography

Radiographs of four dogs with limping, painful lymphangitis and unilateral limb oedema were taken to rule out orthopaedic problems if any. Plain lateral radiographs of metacarpal region revealed no break in the continuity of bone indicating absence of orthopaedic abnormalities. Swelling of soft tissue could be clearly visualized in the radiographs (Plate 11).

4.6. CLINICAL PATHOLOGY

4.6.1. Haematobiochemical Analysis

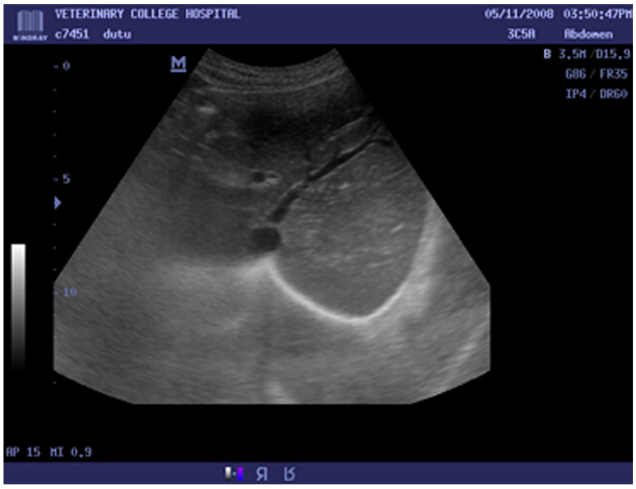
4.6.1.1. Nonsheathed Microfilariae

4.6.1.1.1. Haemogram

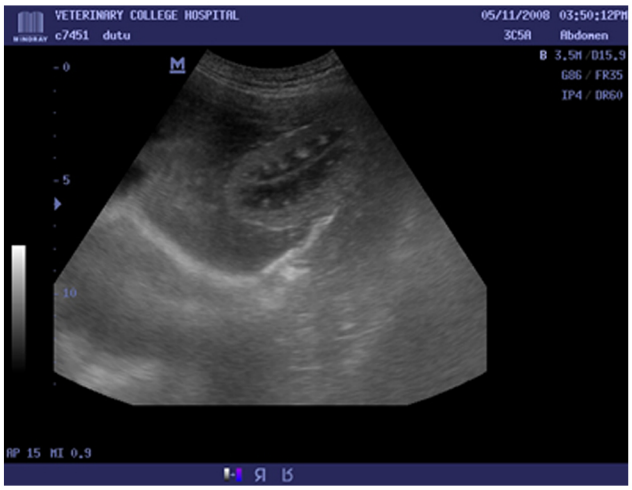
Results of the haemogram are presented in Table 6 : a and b.

The mean total erythrocyte count, haemoglobin and volume of packed red cells in healthy controls were 7.02 ± 0.46 mill/mm^3, 14.03 ± 0.80 g% and 38.13±2.89% respectively. In dogs of group I, II, III, IV and V, the values of total erythrocyte count on day 1 were 5.62 ± 0.50, 5.89 ± 0.73, 5.83 ± 0.43, 6.96 ± 0.38 and 5.91 ± 0.37mill./mm^3 respectively and their corresponding values on day 7 were 5.80 ± 0.55, 6.31 ± 0.51, 6.72 ± 0.43, 6.98 ± 0.61 and 6.4 ± 0.20 mill/ mm^3 respectively. The mean values of haemoglobin in dogs of group I, II, III, IV and V were 11.57 ± 0.99, 12.13 ± 1.02, 12.62 ± 0.96, 14.5 ± 0.86 and 13.05 ± 0.79 g% respectively and their corresponding values on day 7 were 12.53 ± 1.23, 13.55 ± 0.57, 14.00 ± 1.00, 14.70 ± 0.75 and 14.25 ± 0.49 g% respectively. In dogs of group I, II, III, IV and V, the mean pre treatment values of volume of packed red cells were 35.47 ± 3.29, 37.47 ± 3.48, 36.45 ± 3.07, 44.57 ±4.50 and 39.02 ± 2.60% and corresponding post treatment values were 39.03 ± 4.26, 42.13 ± 1.23, 40.67 ± 2.95, 44.73 ± 2.07 and 43.00 ± 1.71 respectively. No statistically significant differences were noticed between the diseased groups and healthy controls. In all the groups, though not significant, an increase was

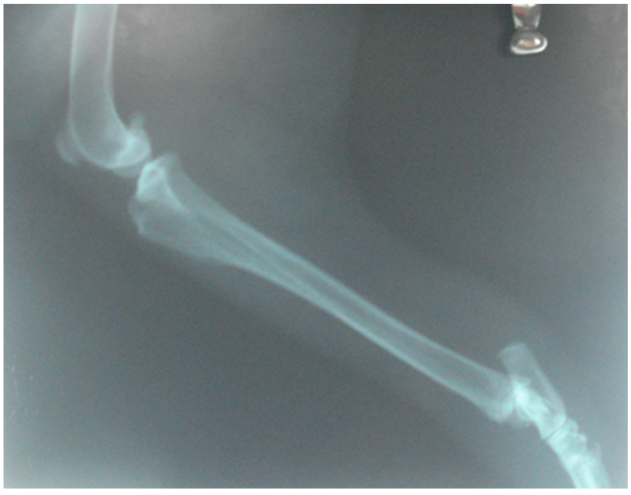
Plate 11: Imaging techniques



A. Ultrasonogram - Liver -focal hyperechoic areas with dilated hepatic vessels



B. Ultrasonogram- Kidney-hyperechoic cortex



C. Radiograph of limb oedema -Normal bone with soft tissue swelling

Table 7 a: Leucogram and platelet count of control dogs (mean ± SE)

Total leucocyte count(/mm3)	Neutrophils(%)	Lymphocytes(%)	Eosinophils(%)	Platelet count(10^3/mm^3)
13900 ± 516.40	77.17 ± 1.94	21.83 ± 4.58	1.00 ± 0.33	373 ± 38.95

Table 7 b: Leucogram and platelet count of dogs of Group I, II, III, IV and V on day1 and 7 of therapeutic trials

Group	Total leucocyte count(/mm3)		Neutrophils (%)		Lymphocytes (%)		Eosinophils(%)		Platelet count(10^3/mm^3)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
I	19283.33^{A} ± 1590.47**	13766.67^{B} ± 687.35	67.17 ± 4.33	81.33 ± 2.09	22 ± 3.2	16 ± 1.69	8.33^{A} ± 3.14**	2.0^{B} ± 1.00	254.17^{A} ± 32.19*	327.75^{B} ± 38.03
II	20416^{A} ± 1320.47**	12671^{B} ± 764.00	74.17 ± 1.72	75.5 ± 2.86	23.33 ± 1.36	23.83 ± 2.57	4.17^{A} ± 0.7*	0.50^{B} ± 0.34	206.33^{A} ± 42.81**	249.00^{B} ± 30.63
III	17433.33^{A} ± 1708.90**	13350^{B} ± 802.04	73.67 ± 5.92	74 ± 5.47	23.33 ± 4.74	24.33 ± 4.96	4.83^{a} ± 1.38*	1.33^{b}± 0.61	223.17^{A} ± 30.23**	284.83^{B} ± 27.42
IV	16926.67^{A} ± 1317.88**	10933.33^{B} ± 803.49	63.33 ± 5.2	73.67± 3.27	27.83 ± 5.9	22 ± 1.77	8.50^{A} ± 2.29**	3.83^{B} ± 1.97	254.67^{A} ± 44.71*	303.83^{B} ± 41.38
V	16783.33^{A} ± 1713.36**	13916^{B} ± 614.47	72.33 ± 4.33	67.5 ± 8.21	22.33 ± 2.63	18.33 ± 3.76	8.17^{A} ± 2.20**	3.33^{B} ± 0.99	282.00^{A} ± 48.13*	318.33^{B} ± 41.96

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

observed in the post treatment values of total erythrocyte count, haemoglobin and volume of packed red cells as compared with the pre treatment values.

The erythrocyte sedimentation rate (ESR) in healthy controls was 3.00 ± 0.37 mm/hr. The mean values of erythrocyte sedimentation rate in dogs of group I, II, III, IV and V were 8.83 ± 4.38, 3.83 ± 0.60, 4.67 ± 1.10, 4.83 ±1.49 and 7.83 ± 2.83 mm/hr respectively. A statistically significant ($P \leq 0.01$) increase in ESR was observed on day 1 in animals of group I and V and also in other three groups ($P \leq 0.05$) when compared to normal value. The values on day 7 became significantly reduced ($P \leq 0.01$) in animals of group II and V and also in animals of group I ($P \leq 0.05$) and was comparable to normal value (Fig.6).

4.6.1.1.2. Leucogram

Results of the leucogram are presented in Table 7 : a and b.

Mean total leucocyte count (TLC) in healthy controls was 13900 ± 516.40/mm^3. In animals of group I, II, III, IV and V, the values of total leucocyte count were 19283.33 ± 1590.47, 20416 ± 1320.47, 17433.33 ± 1708.90, 16926.67 ± 1317.88 and 16783.33 ± 1713.36/mm^3 respectively. Significant ($P \leq 0.01$) increase was noticed in total leucocyte count on day 1 in animals of group I, II , III, IV and V when compared to normal value and statistically significant ($P \leq 0.01$) decrease in TLC level of 13766.67 ± 687.35 /mm^3 in group I, 12671 ± 764/mm^3 in group II and 13350 ± 802.04/mm^3 in group III, 10933.33 ± 803.49/mm^3 in group IV and 13916 ± 614.47/mm^3 in group V were observed on day 7 and the value became comparable to healthy control (Fig.7).

The mean value of neutrophils, lymphocytes and eosinophils of healthy controls were 77.17 ± 1.94, 21.83 ± 4.58 and 1.00 ± 0.33 % respectively. Non significant neutropenia and lymphocytosis could be observed in animals of all groups on day1 when compared to healthy control. The variations in neutrophils on day7 were not significant, but comparable to normal value. No significant differences in lymphocytes could be observed between the pre and post treatment

Table 6 a: Haemogram of control dogs (mean ± SE)

Total erthrocyte count (mill/mm3)	Haemoglobin (g%)	Volume of packed red cells (%)	Erytrocyte sedimentation rate(mm/hr)
7.02 ± 0.46	14.03 ± 0.80	38.13 ± 2.89	3.00 ± 0.37

Table 6 b: Haemogram of dogs of Group I, II, III, IV and V on day1 and 7 of therapeutic trials

Group	Total erthrocyte count (mill/mm3)		Haemoglobin (g%)		Volume of packed red cells (%)		Erytrocyte sedimentation rate(mm/hr)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
I	5.62 ± 0.50	5.80 ± 0.55	11.57 ± 0.99	12.53 ± 1.23	35.47 ± 3.29	39.03 ± 4.26	$8.83 \pm 4.38^{a**}$	3.17 ± 1.98^{b}
II	5.89 ± 0.73	6.31 ± 0.51	12.13 ± 1.02	13.55 ± 0.57	37.47 ± 3.48	42.13 ± 1.23	$3.83 \pm 0.60^{A*}$	1.67 ± 0.49^{B}
III	5.83 ± 0.43	6.72 ± 0.43	12.62 ± 0.96	14.0 ± 1.00	36.45 ± 3.07	40.67 ± 2.95	4.67 ± 1.10*	3.27 ± 0.94
IV	6.96 ± 0.38	6.98 ± 0.61	14.5 ± 0.86	14.7 ± .75	44.57 ±4.50	44.73 ± 2.07	4.83 ±1.49*	2.17 ± 0.60
V	5.91 ± 0.37	6.4 ± 0.20	13.05 ± 0.79	14.25 ± 0.49	39.02 ± 2.60	43.0 ± 1.71	$7.83 \pm 2.83^{A**}$	2.67 ± 1.15^{B}

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

Fig.6:Average ESR (mm/hr) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

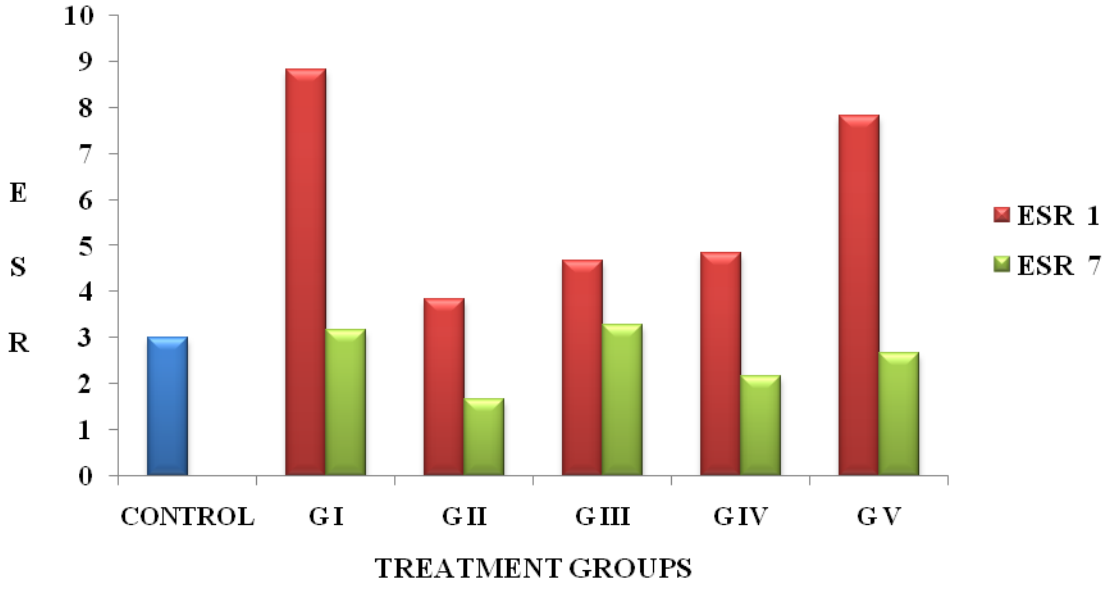
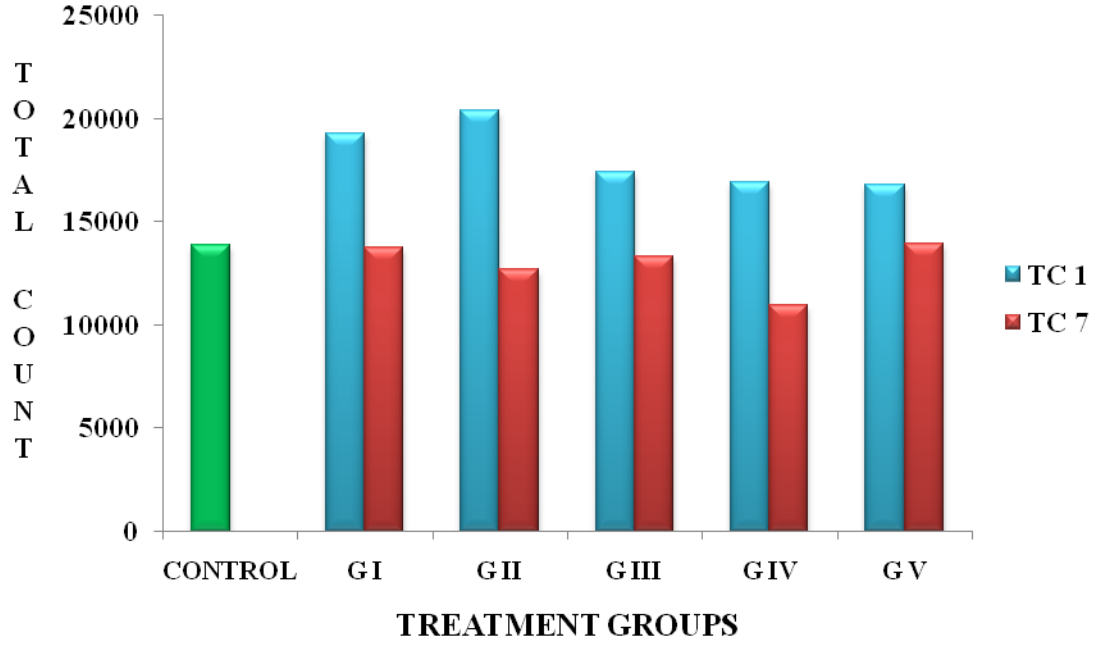


Fig.7:Average total leucocyte count (/mm^3) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.



values. In dogs of group I, II, III, IV and V, the values of eosinophils on day 1 were 8.33 ± 3.14, 4.17 ± 0.70, 4.83 ± 1.38, 8.50 ± 2.29 and 8.17 ± 2.20% respectively. Statistically significant ($P \leq 0.01$) increase in mean eosinophil values was observed on day1 in animals of group I, IV and V and also in group II and III ($P \leq 0.05$). Decrease in eosinophils count of animals of all the five groups on day 7 were statistically significant when compared to its pre treatment values (Fig.8).

4.6.1.1.3. Platelet Count

Results of the platelet count are presented in Table 7 : a and b.

The platelet count of healthy controls were 373 ± 38.95 thousands/mm^3. The mean platelet counts of animals of group I, II, III, IV and V on day1 were 254.17 ± 32.19, 206.33 ±42.81, 223.17 ± 30.23, 254.67 ± 44.71 and 282.00 ± 48.13 thousands/mm^3 respectively and their corresponding values on day7 were 327.75 ± 38.03, 249.00 ± 30.63, 284.83 ± 27.42, 303.83 ± 41.38 and 318.33 ± 41.96 thousands/mm^3 respectively. Statistically significant decrease in platelet counts were noticed on day1 in animals of group II and III ($P \leq 0.01$) and also in other three groups ($P \leq 0.05$). The variations in post treatment values showed a statistically significant ($P \leq 0.01$) increase and comparable to the normal value (Fig. 9).

4.6.1.1.4. Serum total protein, Albumin, Globulin and AG ratio

Results of the serum total protein, albumin, globulin and AG ratio are presented in table 8 : a and b.

The serum total protein, albumin, globulin and AG ratio in healthy controls were 5.85 ± 0.22g/dl, 2.16 ± 0.12g/dl, 3.69 ± 0.16g/dl and 0.60 ± 0.04 respectively. Serum total protein values of animals of group I, II, III, IV and V were 6.45 ± 0.59, 7.18 ± 0.50, 6.35 ± 0.39, 7.44 ± 0.39 and 6.5 ± 0.42 respectively. A significant ($P \leq 0.01$) increase in serum total protein level could be observed in animals of group II and IV on day1 when compared to healthy

Fig.8:Average eosinophils (%) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

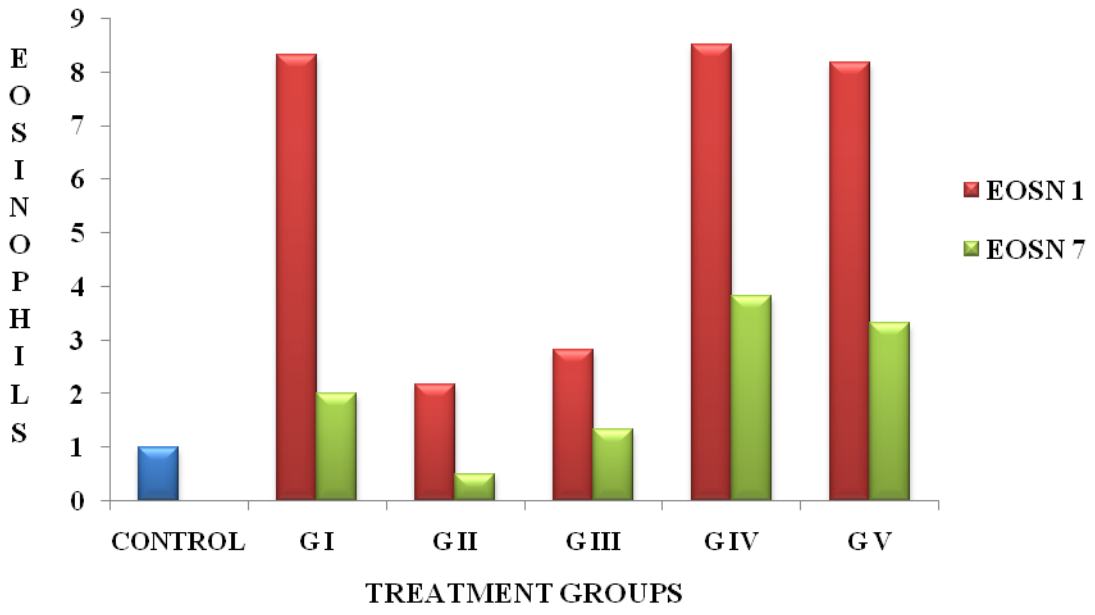


Fig.9:Average platelet count ($10^3/mm^3$) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

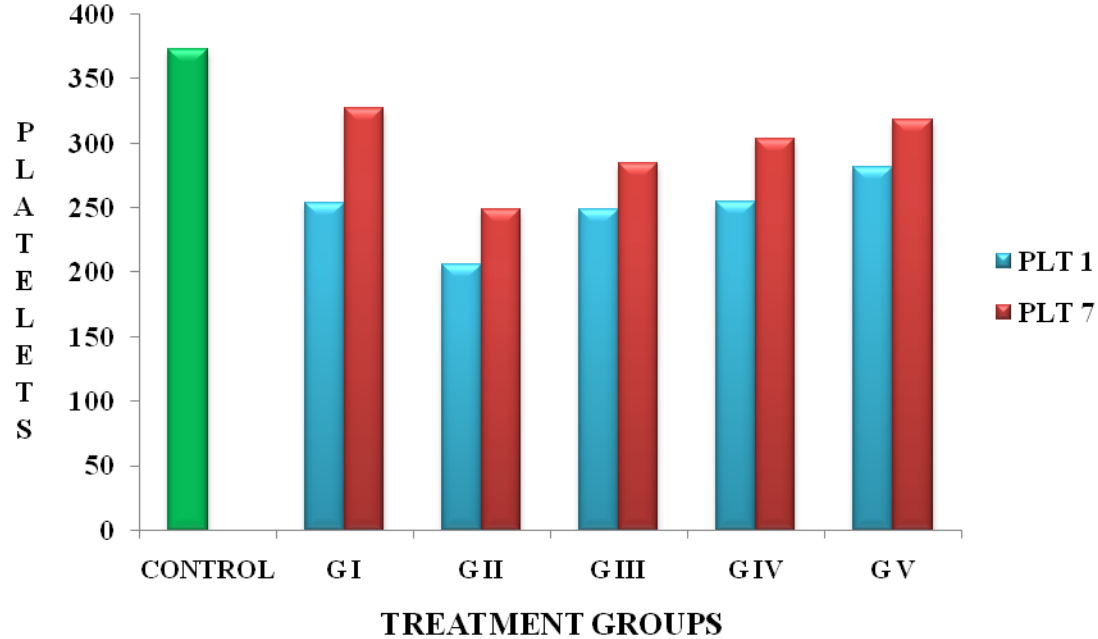


Table 9 a: Serum ALT, AST and ALP values of control dogs (mean ± SE)

Alanine Amino transferase (ALT) (IU/L)	Aspartate Amino transferase (AST) (IU/L)	Alkaline Phosphatase (ALP) (IU/L)
10.50 ± 2.90	24.67 ± 4.94	67.17 ± 17.84

Table 9 b:Serum ALT, AST and ALP values in dogs of Group I, II, III, IV and V on day1 and 7 of therapeutic trials

Group	Alanine Amino transferase (ALT) (IU/L)		Aspartate Amino transferase (AST) (IU/L)		Alkaline Phosphatase (ALP) (IU/L)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
I	71.67 ± 14.57 ^{A}**	52.0 ± 14.16 ^{B}**	56 ± 14.53*	42.17 ± 12.71*	214.42 ± 36.22 ^{A}**	112.24 ± 17.93^{B} *
II	113.50 ± 23.29 ^{A}**	49.83 ± 12.49 ^{B}**	48.0 ± 15.44*	40.33 ± 17.87*	232.06 ± 44.11^{A}**	127.22 ± 24.66 ^{B}*
III	81.50 ± 22.14 ^{A}**	62.67 ± 32.89 ^{B}**	42.17 ± 8.19*	33.33 ± 5.41*	223.96 ± 37.54 ^{A}**	118.35 ± 19.21 ^{B}*
IV	45.00 ± 8.9 ^{A}**	36.00 ± 8.78 ^{A}**	63.00 ± 14.83*	53.83 ± 13.70*	202.01 ± 29.12 ^{A} **	188.78 ± 22.34 ^{A}**
V	61.83 ± 17.09 ^{A}**	35.67 ± 5.39 ^{B}**	92.33 ± 18.25**	77.17 ± 17.04*	236.83 ± 45.26 ^{A}**	131.83 ± 26.7 ^{B}*

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

control group. Non significant increase in serum total protein level were observed in animals of group I, III and V. Animals of all the groups showed non significant reduction in total protein level on day7 and was comparable to normal value (Fig.10). Serum albumin levels in all the five groups of animals during the pre treatment and post treatment periods were not significantly different from control animals. The variations observed during the course of treatment were also statistically non significant.

In animals of group I, II,III, IV and V ,the mean values of globulin on day1 were 4.47 ± 0.54, 5.01 ± 0.53, 4.64 ± 0.29, 5.30 ± 0.25 and 4.68 ± 0.44 g/dl respectively and their corresponding values on day 7 were 3.69 ± 0.31, 3.67 ± 0.16, 3.64 ± 0.49, 4.06 ± 0.32 and 3.95 ± 0.34 g/dl respectively. Statistically significant ($P \leq 0.01$) increase in globulin level was observed in all the five groups on day1 when compared to healthy controls. The level of globulin reduced significantly ($P \leq 0.05$) on day 7 when compared to day 1 and became comparable to the normal level. No significant differences were noticed between the treatment groups (Fig.11).

The AG ratio of animals of group I, II, III, IV and V on day1 were reduced, but not significant when compared to normal value. But significant increase could be observed in AG ratio of all the groups on day 7 when compared to pre treatment values and were comparable to the healthy control group (Fig.12).

4.6.1.1.5. Serum ALT, AST and ALP

Results of serum ALT, AST and ALP are presented in table 9 : a and b.

The mean serum alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase levels in healthy controls were 10.50 ± 2.90, 24.67 ± 4.94 and 67.17 ± 17.84 IU/L respectively. In dogs of group I, II, III, IV and V ,the values ofALT on day 1 were 71.67 ± 14.57, 113.50 ± 23.29, 81.50 ± 22.14, 45.00 ± 8.9 and 61.83 ± 17.09 IU/L respectively and their

Fig.10:Average serum total protein (g/dl) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

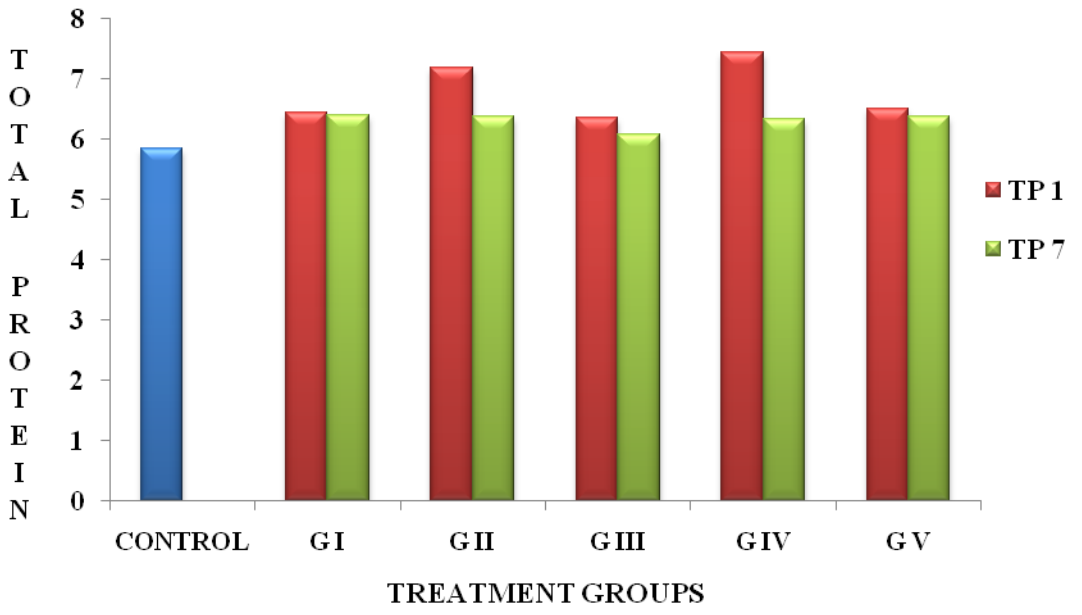


Fig.11: Average serum globulin (g/dl) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

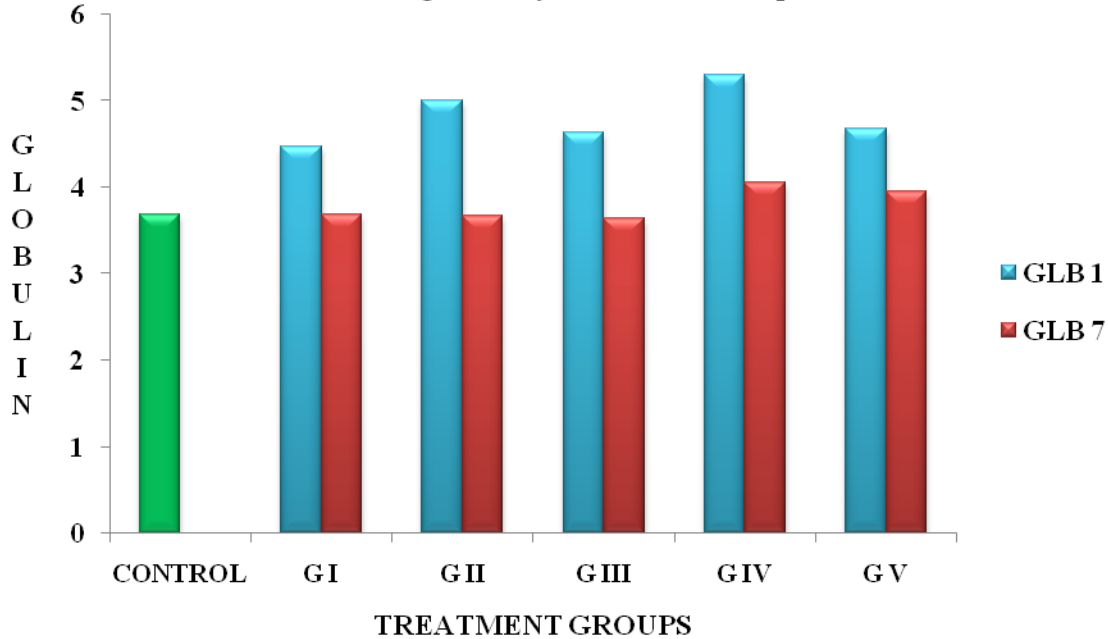


Table 8 a:Serum total protein, Albumin, Globulin and AG ratio values of control dogs (mean ± SE)

Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Albumin Globulin (AG) Ratio
5.85 ± 0.22	2.16 ± 0.12	3.69 ± 0.16	0.60 ± 0.04

Table 8 b:Serum total protein, Albumin, Globulin and AG ratio values of dogs of Group I, II, III, IV and V on day1 and 7 of therapeutic trials

Group	Total Protein (g/dl)		Albumin (g/dl)		Globulin (g/dl)		Albumin Globulin (AG) Ratio	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
I	6.45 ± 0.59	6.39 ± 0.46	1.98 ± 0.15	2.49 ± 0.19	4.47 ± 0.54^{a**}	3.69 ± 0.31^{b}	0.48 ±0.12 ^{a}	0.69 ± 0.03 ^{b}
II	7.18 ± 0.50**	6.37 ± 0.33	2.15 ± 0.29	2.49 ± 0.25	5.01 ± 0.53 ^{a**}	3.67 ± 0.16 ^{b}	0.46 ± 0.10 ^{a}	0.69 ± 0.11 ^{b}
III	6.35 ± 0.39	6.68 ± 0.41	2.30 ± 0.18	2.69 ± 0.13	4.64 ± 0.29 ^{a**}	3.64 ± 0.49 ^{b}	0.58 ±0.13 ^{a}	0.71 ± 0.14 ^{b}
IV	7.44 ± 0.39**	6.34 ± 0.32	2.13 ± 0.29	2.28 ± 0.25	5.30 ± 0.25 ^{a**}	4.06 ± 0.32 ^{b}	0.41 ±0.03 ^{a}	0.55 ± 0.10 ^{b}
V	6.5 ± 0.42	6.08 ± 0.27	2.22 ±0.36	2.60 ± 0.15	4.68 ± 0.44 ^{a**}	3.95 ± 0.34 ^{b}	0.51 ± 0.11 ^{a}	0.67 ± 0.21 ^{b}

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

corresponding values on day 7 were 52.0 ± 14.16, 49.83 ± 12.49, 62.67 ± 32.89, 36.00 ± 8.78 and 35.67 ± 5.39 IU/L respectively. The pre treatment values of ALT in all the groups showed a statistically significant ($P \leq 0.01$) increase when compared to healthy controls. A significant ($P \leq 0.01$) decrease could be observed on day 7 in animals of group I, II, III and V when compared to pre treatment values but not comparable to normal value. No significant differences were noticed between the pre and post treatment values of animals of group IV (Fig.13).

The mean AST level of all the groups were statistically significant ($P \leq 0.05$) when compared to healthy controls. In all the groups AST level become reduced by day 7. The reduction in AST level in all the groups on day 7 were not statistically significant when compared to corresponding pre treatment values and was not comparable to normal level also (Fig.14).

In dogs of group I, II, III, IV and V, the values of ALP on day1 were 214.42 ± 36.22, 232.06 ± 44.11, 223.96 ± 37.54, 202.01 ± 29.12 and 236.83 ± 45.26 IU/L respectively. Statistically significant ($P \leq 0.01$) increase in the mean values of ALP were observed on day 1 when compared to healthy controls. A significant ($P \leq 0.01$) decrease in ALP level was observed on day 7 in all groups except group IV when compared to pre treatment values, but not comparable to normal level. No significant differences were noticed between the pre and post treatment values of group IV animals (Fig.15).

4.6.1.1.6. Blood Urea Nitrogen and Creatinine

Results of blood urea nitrogen and creatinine are presented in table 10 : a and b.

The blood urea nitrogen (BUN) and creatinine levels in healthy controls were 16.29 ± 3.46 and 0.33 ± 0.09 mg/dl respectively. The mean values of BUN and creatinine of group I, II, III, IV and V were 35.07 ± 7.99, 37.69 ± 8.56, 32.49 ± 6.23, 35.86 ± 7.35 and 44.36 ± 8.71mg/dl respectively. The animals of all the

Fig.12: Average albumin globulin ratio in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

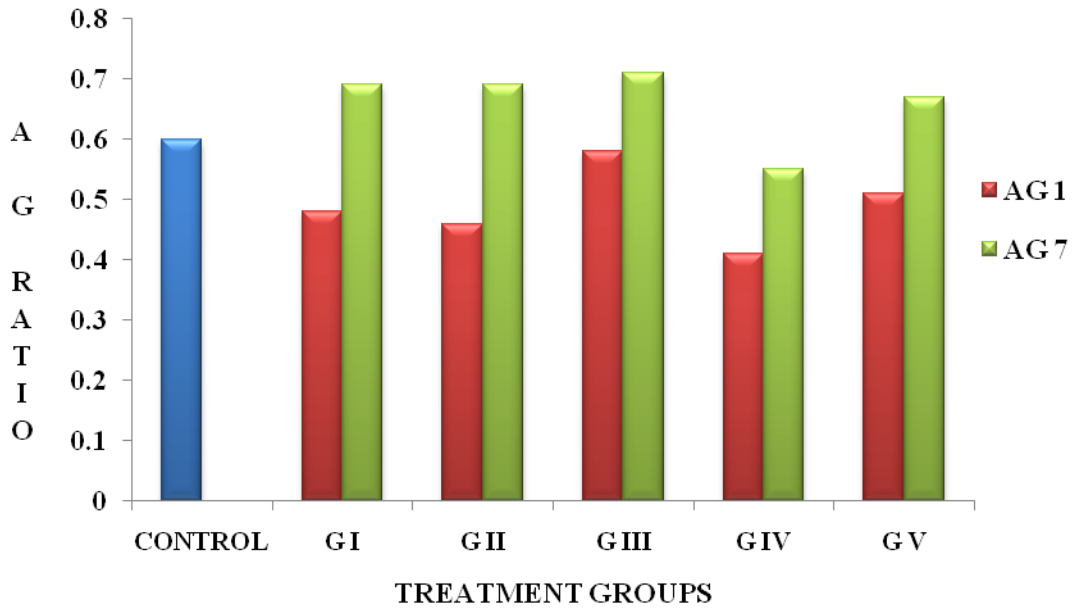


Fig.13:Average serum ALT in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

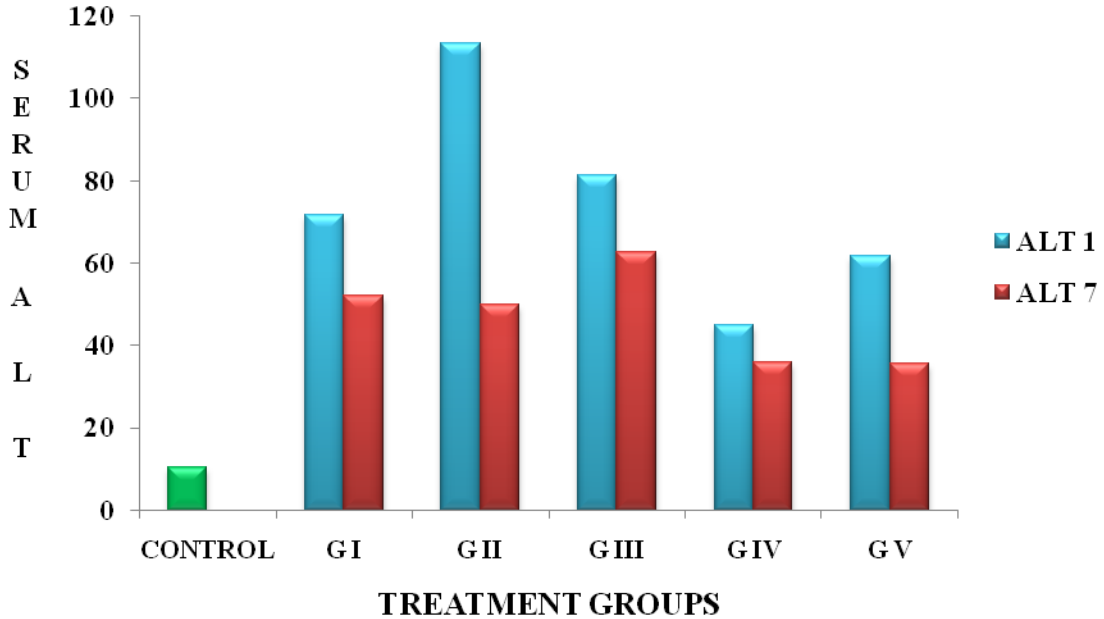


Fig.14:Average serum AST in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

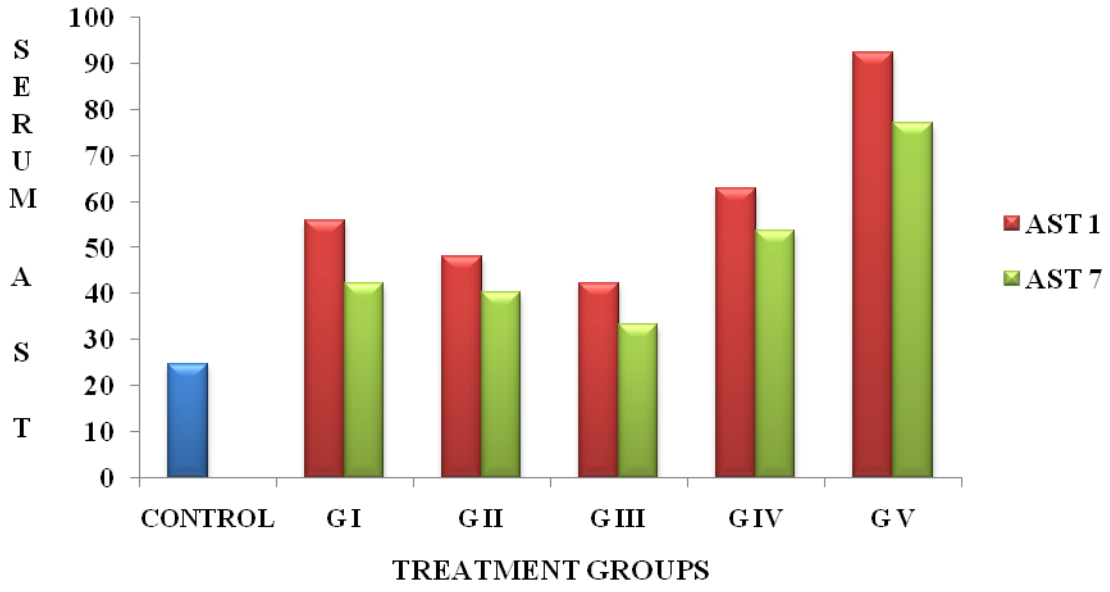


Fig.15:Average serum ALP in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

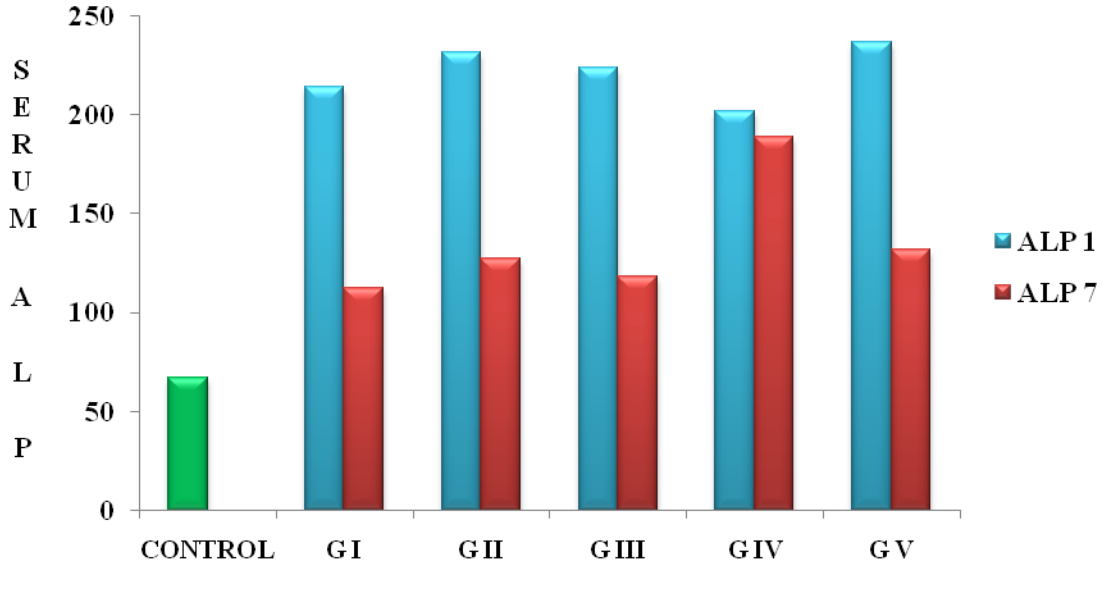


Table 10 a: BUN and Creatinine of control dogs (mean ± SE)

Blood Urea Nitrogen (mg/dl)	Creatinine (mg/dl)
16.29 ± 3.46	0.33 ± 0.09

Table 10 b: BUN and Creatinine values of dogs of Group I, II, III, IV and V on day1 and 7 of therapeutic trials

Group	Blood Urea Nitrogen (mg/dl)		Creatinine (mg/dl)	
	Day 1	Day 7	Day 1	Day 7
I	35.07 ± 7.99 ^{a*}	18.37 ± 4.87 ^{b}	2.22 ± 0.53 ^{a**}	1.11 ± 0.16 ^{b*}
II	37.69 ± 8.56 ^{a*}	30.18 ± 9.48 ^{b}	2.58 ± 1.06 ^{a**}	1.64 ± 0.47 ^{b**}
III	32.49 ± 6.23 ^{a*}	19.72 ± 1.64 ^{b}	4.44 ± 0.81 ^{a**}	2.27 ± 0.15 ^{b**}
IV	35.86 ± 7.35 ^{a*}	31.93 ± 6.9 ^{a}	2.36 ± 0.76 ^{a**}	2.28 ± 0.52 ^{a**}
V	44.36 ± 8.71 ^{a*}	29.81 ± 8.64 ^{b}	3.13 ± 0.44 ^{A**}	1.38 ± 0.11 ^{B}

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

five groups showed significant (P≤0.05) increase in BUN value on day1 when compared to healthy control .The corresponding post treatment values in group I, II, III and V showed a significant (P≤0.05) decrease when compared with values on day1 and was not comparable to normal value. Animals of group IV showed no statistically significant differences between the pre and post treatment values (Fig.16).

The average creatinine level in dogs of group I, II, III, IV and V were 2.22 ± 0.53, 2.58 ± 1.06, 4.44 ± 0.81, 2.36 ± 0.76 and 3.13 ± 0.44 mg/dl respectively. The creatinine level of all the five group of animals on day1 was significantly (P≤0.01) increased. The creatinine level of animals of group V on day 7 was significantly (P≤0.01) reduced than corresponding values of animals of group I, II and III (P ≤ 0.05). The reduction in serum creatinine level in animals of group V on day 7 was comparable to normal level while the animals of other groups did not return to normal level on day 7. No statistically significant differences were observed between the values on day1 and day7 of group IV animals (Fig.17).

4.6.1.2. Sheathed Microfilariae

4.6.1.2.1. Haemogram, Leucogram and Platelet

Results of the haemogram are presented in table 11.

Total leucocyte count of healthy controls and day 1 and 7 of animals of group VI were 13900.0 ± 516.40, 15733.33 ± 1231.71 and 14133.33 ± 895.05/mm3 respectively. Statistical analysis showed significant (P≤0.05) increase in leucocytic count on day1 when compared to healthy controls. The mean value of total count on day 7 was significantly (P≤0.05) reduced when compared to day 1. Differential leucocyte count showed significant (P≤0.05) increase in eosinophil count on day 1 when compared to control group and value became significantly (P ≤ 0.05) reduced on day 7.

Fig.16:Average blood urea nitrogen (mg/dl) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

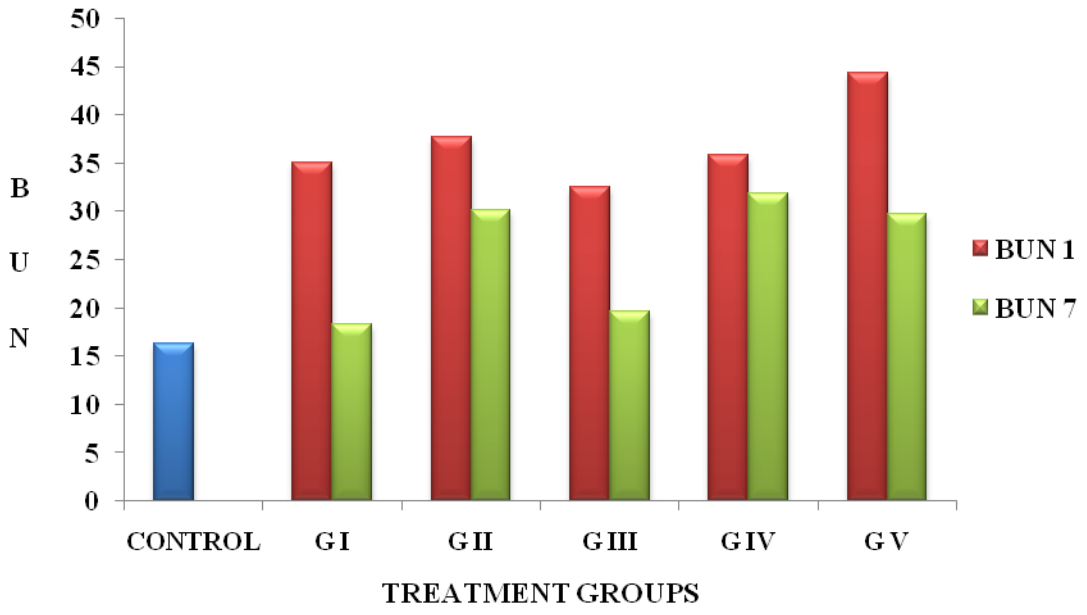


Fig.17:Average serum creatinine (mg/dl) in control and microfilaraemic dogs on day 1 and 7 of therapeutic trials.

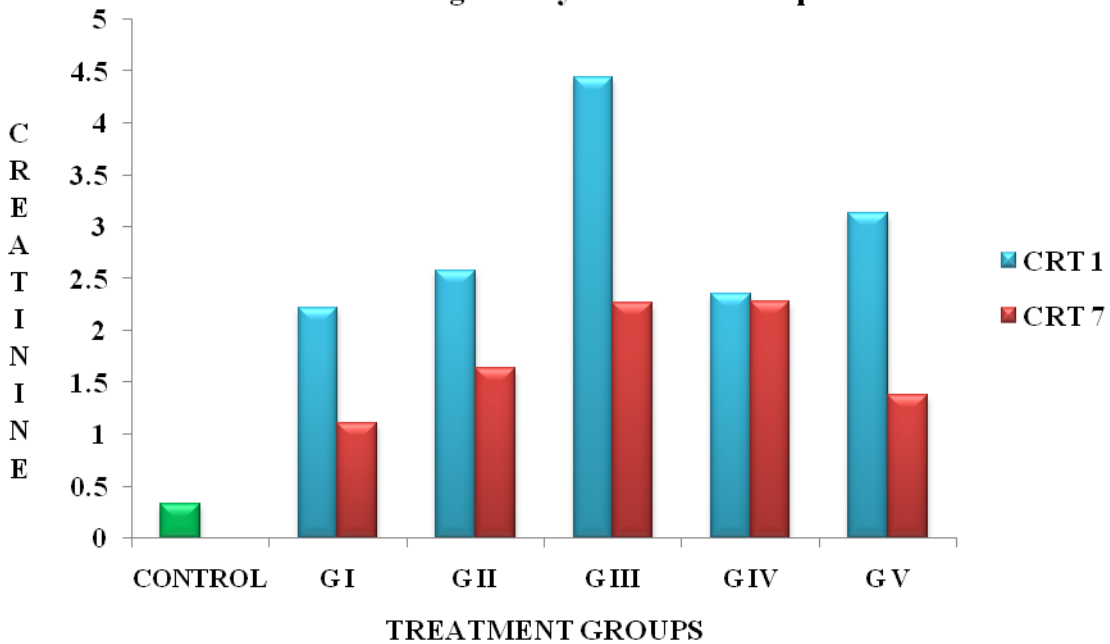


Table 11. Haemogram, leukogram and platelet count of control and group VI dogs on day1 and 7 of therapeutic trials

Parameters	Control	Group VI	
		Day 1	Day 7
Total count(/mm^3)	13900.0 ± 516.40	15733.33 ± 1231.71^{a}*	14133.33 ± 895.05^{b}
Neutrophils(%)	77.17 ± 1.94	68.83 ± 4.60	72.83 ± 4.79
Lymphocytes(%)	21.83 ± 4.58	25.67 ± 3.65	25 .00 ± 4.44
Eosinophils(%)	1.00 ± 0.333	5.50 ± 2.03 ^{a}*	2.17 ± 0.83^{b}
Haemoglobin(g%)	14.033 ±	13.9 ± 0.598	13.9 ± 0.5
Erythrocyte count (mill/mm^3)	7.017 ± 0.464	6.6 ± 0.2527	6.717 ± 0.23
Volume of packed red cells (%)	38.133 ± 2.894	38.08 ± 1.363	41.50 ±1.20
Erythrocyte sedimentation rate (mm/hr)	3.00 ± 0.37	10.50 ± 5.93^{a}**	8.83 ± 6.46^{b}**
Platelet count (10^3/mm^3)	373 ± 38.95	273.83 ± 30.32^{A}*	330.17 ± 36.51^{B}

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

The erythrocyte sedimentation rate (ESR) in healthy controls and day 1 and 7 of animals of group VI were 3.00 ± 0.37, 10.50 ± 5.93 and 8.83 ± 6.46mm/hr. A significant (P≤0.01) increase in ESR was observed on day 1 when compared to healthy controls. The value on day 7 became reduced significantly (P≤0.05), but not comparable to normal level.

Mean platelet count of healthy controls and day 1 and 7 of animals of group VI were 373 ± 38.95, 273.83 ± 30.32 and 330.17 ± 36.51 thousands/mm^3 respectively. Statistical analysis revealed significant (P≤0.05) decrease in thrombocyte count on day 1 when compared to control animals and the value became significantly (P≤0.01) increased on day 7 and was comparable to normal level. The mean values of haemoglobin, VPRC, RBC and neutrophils and lymphocyte percentage were not statistically significant in normal and diseased animals (Fig.18).

4.6.1.2.2. Serum Biochemical Analysis

Results of serum biochemical analysis are presented in table 12

Total protein, albumin, globulin and A:G ratio in healthy controls were 5.85 ± 0.22, 2.16 ± 0.2, 3.69 ± 0.16 g/dl and 0.59 ± 0.04 respectively. Corresponding values of dogs of group VI on day 1 were 8.10 ± 0.30, 2.61 ± 0.21, 5.49 ± 0.63 g/dl and 0.49 ± 0.05 respectively. Statistical analysis revealed significant (P≤0.01) increase in serum total protein and globulin on day 1 with non significant reduction in A:G. Statistically significant (P≤0.01) reduction in total protein and globulin with non significant increase in A:G ratio could be observed on day 7 when compared to day 1. Serum ALT activity of healthy controls and day 1 and 7 of animals of group VI were 10.50 ± 1.02, 47.67± 6.91 and 30.0 ± 3.62 IU/L respectively. Statistical analysis showed significant (P≤0.01) increase in ALT activity on day 1 and significantly (P≤0.01) reduced on day 7 but it did not reach to the normal level. Mean serum ALP of healthy controls and day1 and day 7 of group VI dogs were 67.17± 17.80, 236.83±45.26

Table 12. Serum biochemistry of control & group VI dogs on day1&7 of therapeutic trials

Parameters	Control	Group VI	
		Day 1	Day 7
Total Protein (g/dl)	5.85 ± 0.22	8.10 ± 0.30^{A}**	6.02 ± 0.22^{B}
Albumin (g/dl)	2.16 ± 0.12	2.61 ± 0.21	2.38 ± 0.12
Globulin (g/dl)	3.69 ± 0.16	5.49 ± 0.63 ^{A}**	3.64 ± 0.23^{B}
A:G Ratio	0.59 ± 0.04	0.49 ± 0.05	0.64 ± 0.05
Alanine Amino transferase (ALT)(IU/L)	10.50 ± 1.02	47.67 ± 6.91^{A}**	30 ± 3.62 ^{B}**
Aspartate Amino transferase (AST)(IU/L)	24.67 ± 4.94	43.83 ± 11.31 *	37.67 ± 7.48 *
Alkaline Phosphatase(IU/L)	67.17 ± 17.80	236.83 ± 45.26^{A}**	131.83 ± 26.70 ^{B}**
Blood Urea Nitrogen(mg/dl)	16.29 ± 3.46	33.84 ± 5.10^{a}*	27.84 ± 4.80 ^{a}
Creatinine (mg/dl)	0.33 ± 0.09	2.35± 0.77^{a}*	1.32 ± 0.31^{b}**

**- Significant $p \leq 0.01$ and *-Significant $p \leq 0.05$ when compared with control

Means within the same row of the same parameter with different superscript differ

A, B - $p \leq 0.01$ a, b - $p \leq 0.05$

and 131.83±26.70IU/L respectively. Significant (P≤ 0.01) increase could be observed in the level of ALP on day 1 when compared to healthy control and the value reduced significantly (P≤0.01) on day 7 but did not reach the normal level. The mean AST level of animals of group VI increased significantly (P ≤ 0.05) on day 1 when compared to healthy controls and its level reduced by day 7. The reduction in AST level on day 7 were not statistically significant when compared to the pre treatment value.

Mean serum creatinine value of healthy controls and day1 and 7 of dogs of group VI were 0.33± 0.09, 2.35±0.77 and 1.32±.31 mg/dl respectively. Significant increase (P≤ 0.05) in creatinine value could be observed on day 1 when compared to normal value and value was significantly (P≤0.05) reduced on day 7, but not comparable to normal value. Mean blood urea nitrogen concentration of healthy control and day 1 and 7 of group VI animals were 16.29±3.46, 33.84±5.10 and 27.84±4.82 mg/dl respectively. Statistical analysis showed significant (P≤ 0.05) increase in mean value of BUN on day 1 when compared to healthy dogs and value became reduced on day 7, but not statistically significant (Fig.19 and 20).

4.6.2. Urinalysis

4.6.2.1. Qualitative Analysis

Qualitative urinalysis revealed the presence of urine protein (4+), bilirubin, blood pigments and bile pigments with a mean specific gravity of 1.010 (1.005-1.020).

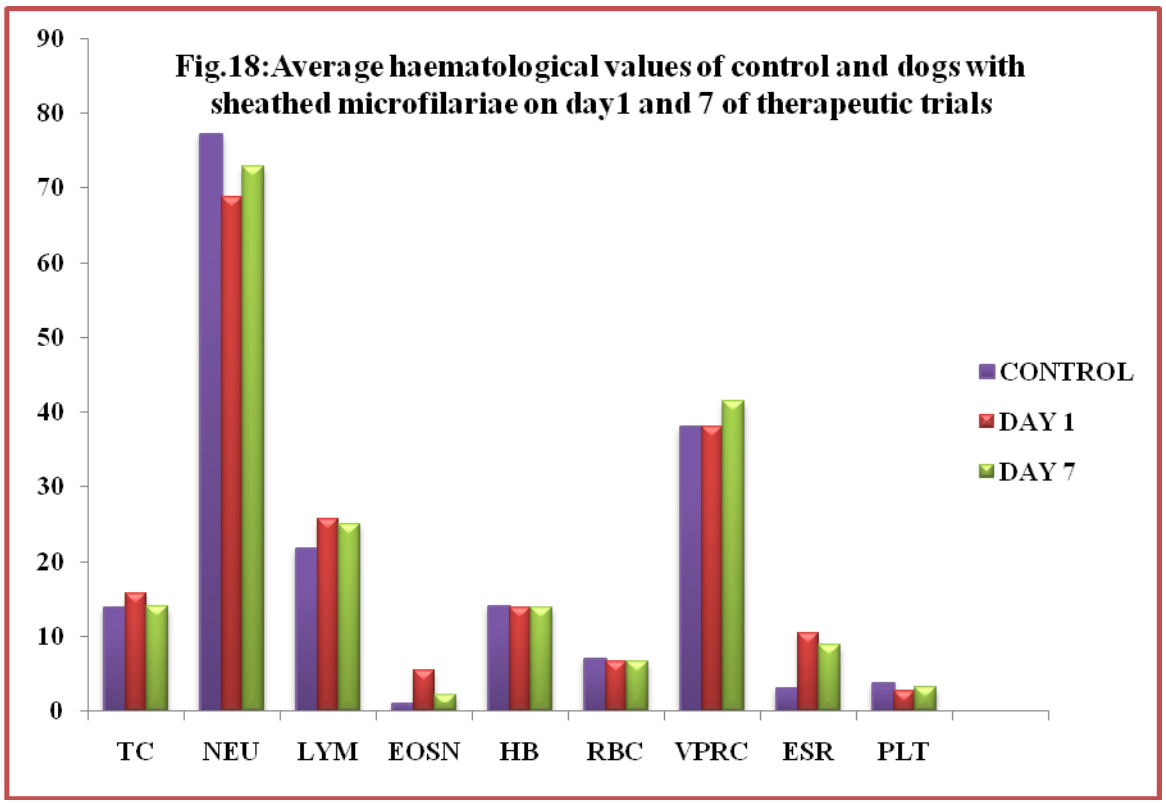


Fig.18:Average haematological values of control and dogs with sheathed microfilariae on day1 and 7 of therapeutic trials
90
80
70
60
50
40
30
20
10
0
TC
NEU
LYM
EOSN
HB
RBC
VPRC
ESR
PLT
CONTROL
DAY 1
DAY 7

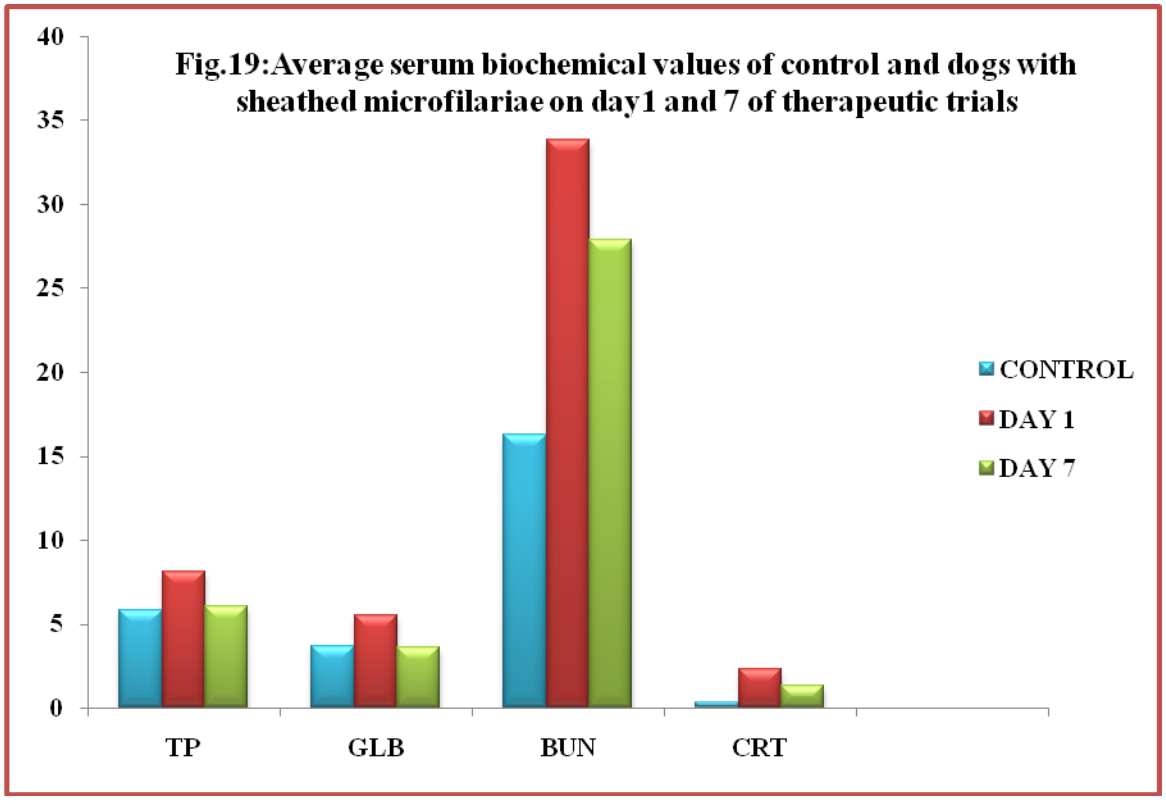


Fig.19:Average serum biochemical values of control and dogs with sheathed microfilariae on day1 and 7 of therapeutic trials
40
35
30
25
20
15
10
5
0
TP
GLB
BUN
CRT
CONTROL
DAY 1
DAY 7

4.6.2.2. Quantitative Analysis

Table 13. Urinalysis of control and day 1 of microfilaraemic dogs

Parameters	Control	Microfilaraemic animals
N-Acetyl-β-D-Glucosaminidase (NAG) (U/g of creatinine)	7.08 ± 1.00	17.54±1.70**
Urine Protein Creatinine Ratio (UPC)	0.38 ± 0.05	1.91±0.67*
γ-Glutamyl transferase (GGT) (IU/L/mmol creatinine)	1.02 ± 0.42	1.81±0.59*
Alkaline phosphatase (ALP) (IU/L/mmol creatinine)	5.78 ±1.57	15.79±8.40*

The mean values of NAG, UPC, GGT and ALP were 7.08 ± 1.00 U/g of creatinine, 0.38 ± 0.05, 1.02 ± 0.42 and 5.78 ±1.57 IU/L/mmol creatinine respectively in healthy controls. Urinalysis revealed significant increase in NAG (17.54±1.70 U/g of creatinine), UPC (1.91±0.67), γ GT (1.81±0.59 IU/L/mmol creatinine) and ALP (15.79±8.40 IU/L/mmol creatinine) on day 1 of microfilaraemic dogs when compared to non microfilaraemic controls (Table 13 and Fig.21).

Results of the present study revealed leucocytosis with eosinophilia and thrombocytopenia in microfilaraemic dogs. Elevated levels of serum total protein, globulin, serum enzymes like ALT and ALP and nonsignificant reduction in AG ratio suggestive of liver pathology in microfilaraemic dogs. Elevated levels of BUN, creatinine, urine protein creatinine ratio, NAG, ALP, proteinuria with low specific gravity confirmed the renal involvement in diseased animals.

Fig.20:Average serum ALT, AST and ALP of control and dogs with sheathed microfilariae on day1 and 7 of therapeutic trials

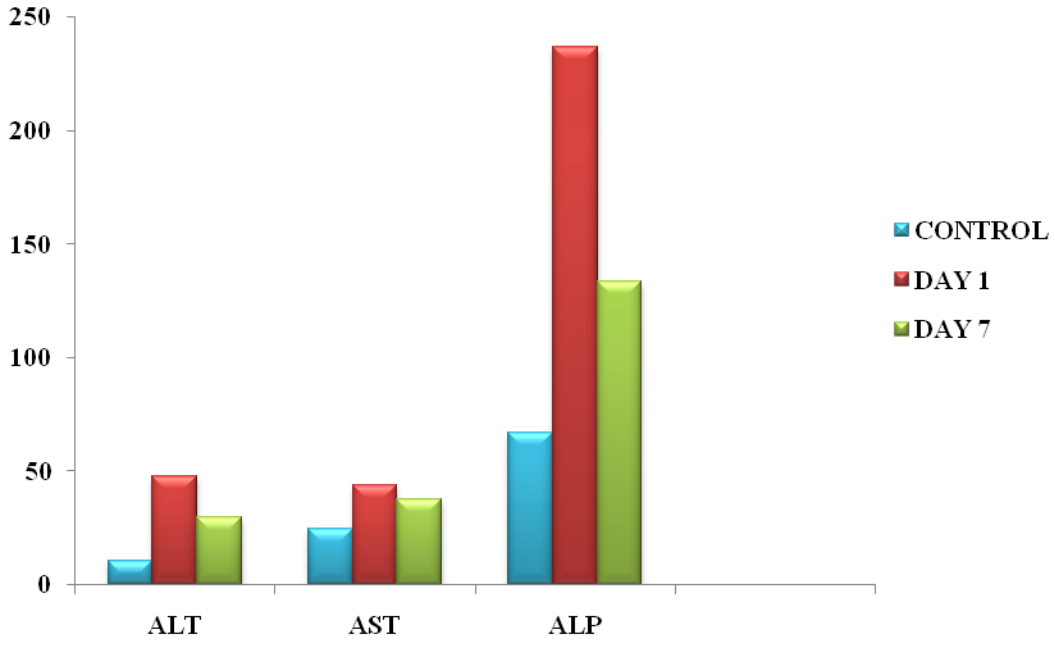
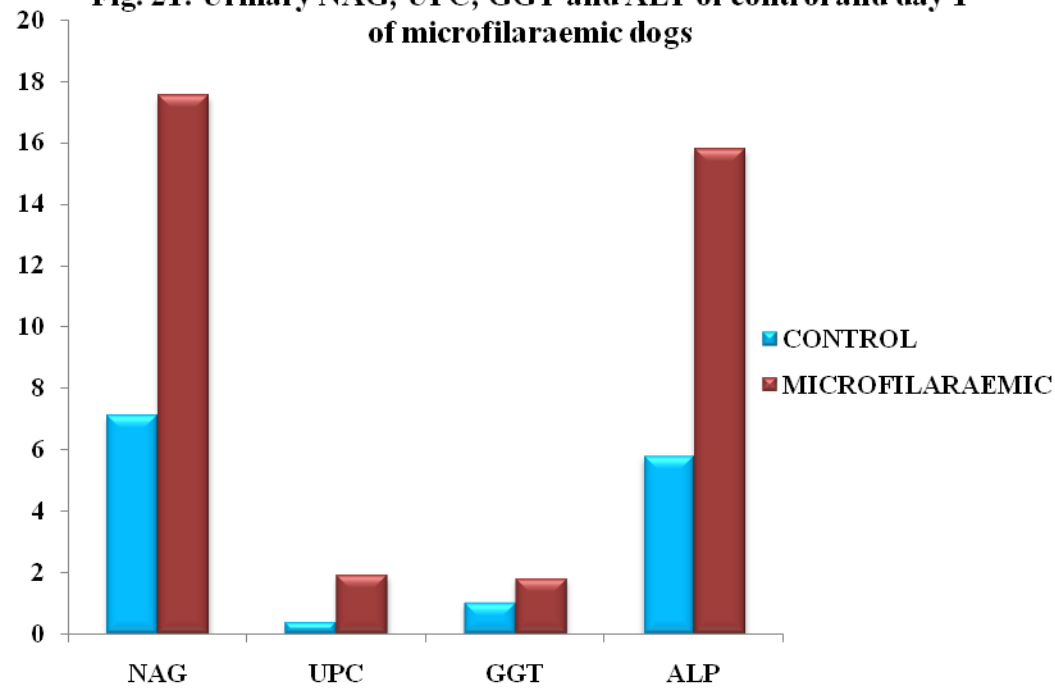


Fig. 21: Urinary NAG, UPC, GGT and ALT of control and day 1 of microfilaraemic dogs



4.7. TREATMENT

4.7.1. Clinical Response

4.7.1.1. Nonsheathed Microfilariae

Table 14. Number and percentage amicrofilaraemia in different days of treatment

Groups (10 each)	Day 2		Day 3		Day 4		Day 7	
	Number	Per cent	Progre-ssive total	Per cent	Progre-ssive total	Per cent	Progre-ssive total	Per cent
I	5	50	7	70	10	100	0	0
II	5	50	10	100	0	0	0	0
III	3	30	7	70	10	100	0	0
IV	0	0	0	0	0	0	0	0
V	2	20	5	50	8	80	10	100

The clinical response was evaluated by the periodic clearance of microfilariae on wet blood film examination. In group I, 50% of animals cleared off microfilariae by day 2 and 70% on day 3 and 100% on day 4. In group II, 50% each of animals cleared off microfilariae on day 2 and day 3. Complete clearance of microfilariae could be observed in animals of group II by day 3. Thirty per cent dogs were negative for microfilaraemia on day 2 and 70% on day 3 and complete clearance by day 4 in group III. No periodic clearance of circulating microfilaria could be observed in dogs of group IV even after seven days of treatment. The percentage clearance rate in animals of group V were 20, 50, 80 and 100% 0n day 2, 3, 4 and 7 respectively (Table 14 and Fig.22).

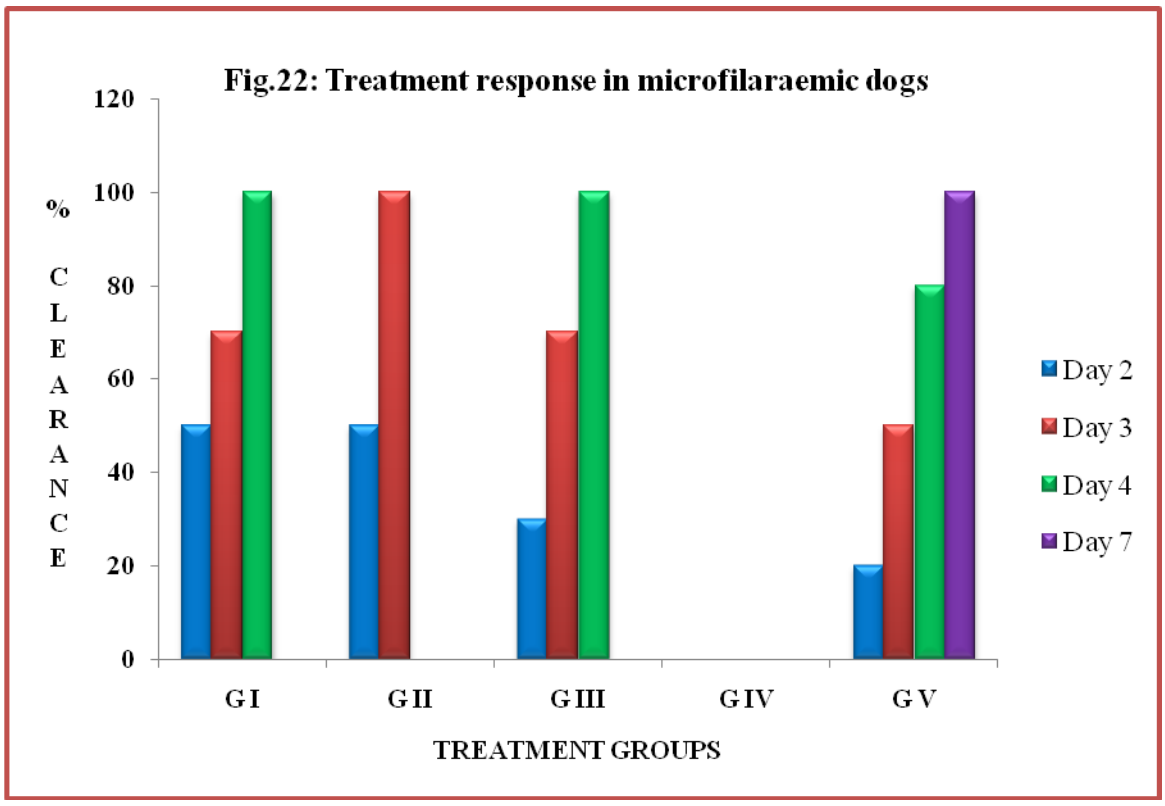


Fig.22: Treatment response in microfilaraemic dogs
120
100
80
60
40
20
0
% CLEARANCE
G I
G II
G III
G IV
G V
TREATMENT GROUPS
Day 2
Day 3
Day 4
Day 7

4.7.1.2. Sheathed Microfilariae

Table 15. Number and percentage amicrofilaraemia in different days of treatment

Group (16)	Day 2		Day 3		Day 4	
	Number	Per cent	Progressive total	Per cent	Progressive total	Per cent
VI	3	18.75	7	43.75	16	100

In group VI, 18.75 % of dogs were cleared off microfilaraemia by day 2 followed by 43.75 on 3rd and 100% on 4th day of treatment respectively.

4.7.2. Treatment Response

4.7.2.1. Nonsheathed Microfilariae

In all the groups except group IV, animals responded very well to the concerned treatment. Remission of clinical signs was observed in all the 40 cases treated in group I, II, III and V. But recurrence was reported in one case of group V after one month of treatment. Clinical signs and microfilaraemia persists as such in eight animals of group IV even after 7 days of treatment and two animal of this group died during the course of therapy.

4.7.2.2. Sheathed Microfilariae

All the animals of group VI were successfully treated with levamisole hydrochloride @ 10 mg/kg body weight for seven days since initial treatment as in case of group I, II, III and IV were not satisfactory. Clinical signs like anorexia, vomiting, fever, conjunctivitis and scrotal oedema showed complete remission. In one animal limb oedema was reduced much, but still persisted even after 7 days of treatment. Lymphangitis persisted in all the 5 animals even after the animal became amicrofilaraemic.

4.7.3. Adverse Drug Reactions

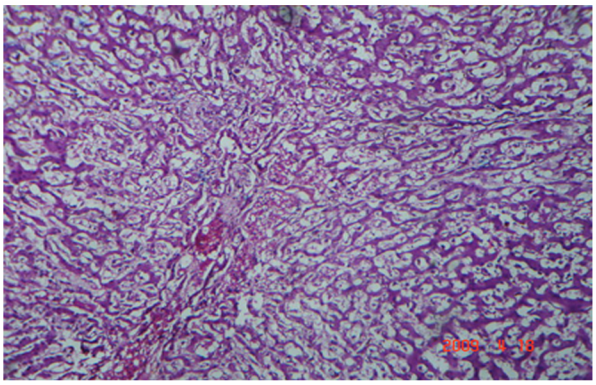
Erratic drug reactions like depression, ataxia and drowsiness were observed in four animals treated with single oral dose of ivermectin @ 200 µg/kg body weight. Six animals received treatment with levamisole hydrochloride @ 10 mg/kg body weight orally for seven days, showed vomiting.

4.8. AUTOPSY AND HISTOPATHOLOGY

Two animals died during the course of treatment were subjected to postmortem examination. The gross pathological lesions observed were fibrosis and congestion of liver, congestion of lungs and alveolar emphysema, cardiac hypertrophy and small and pale kidneys with granular pitted surface. An adult worm of about 15cm length was retrived from the subcutaneous tissue of one animal and identified as *Dirofilaria repens.*

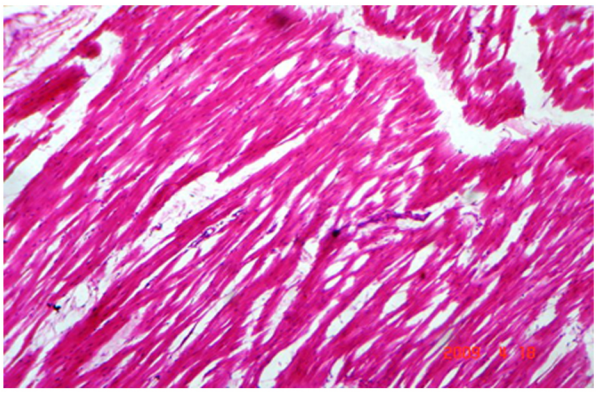
Histopathologically the heart revealed myofibrillar fragmentation and lungs with atlectasis, congestion of capillaries and thromboemboli formation. Hepatic lesions included passive congestion, cirrhosis and portal hepatitis (plate 12). The kidneys revealed vacuolation, necrosis and predominantly hyalinization and atrophy of glomeruli with the presence of dense granular deposits adjacent to the glomeruli, diffuse tubular necrosis with desquamation of tubular epithelium and fibrous tissue proliferation in the interstitium indicating chronic interstitial nephritis. The renal tubules with hyaline cast indicating the presence of proteinuria (Plate 13).

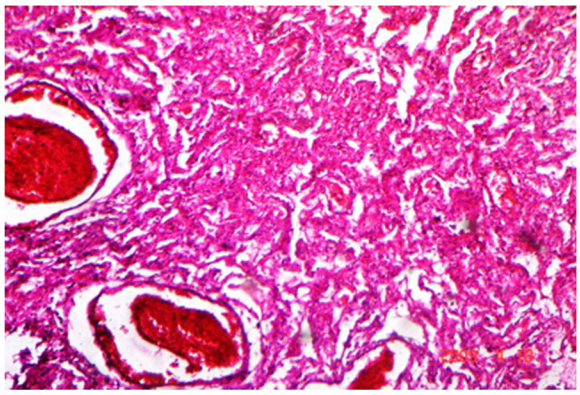
Plate 12: Histopathology



A. Liver-Cirrhosis, fibrous tissue proliferation

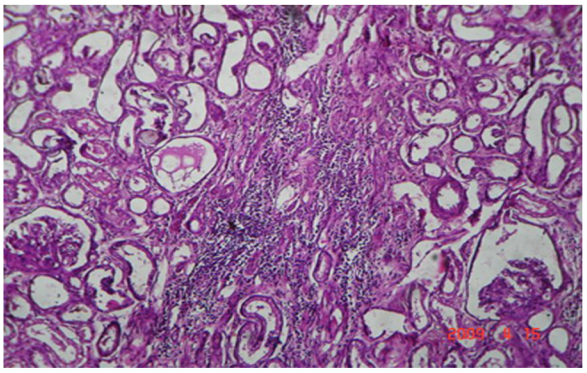
B. Heart - hypertrophy, separation and fragmentation of fibres



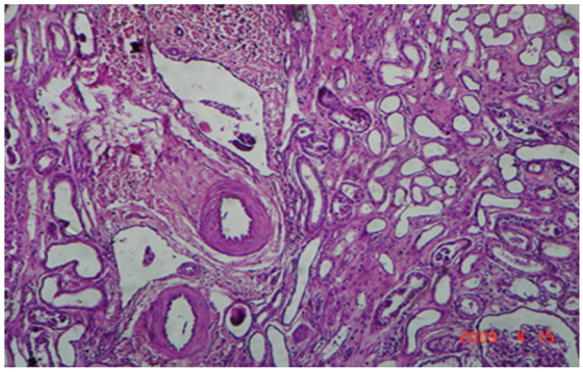


C. Lungs - Atlectasis, congestion of pulmonary vessel,alveolar haemorrhage

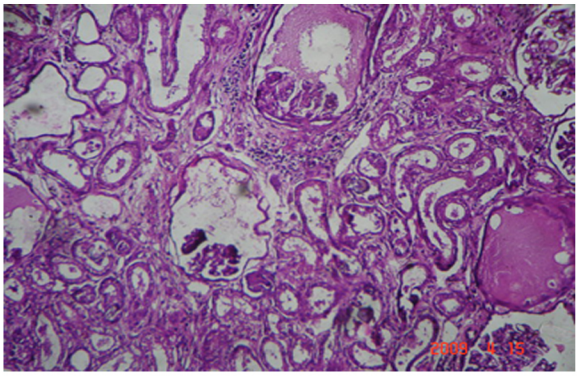
Plate 13: Histopathology- Kidney



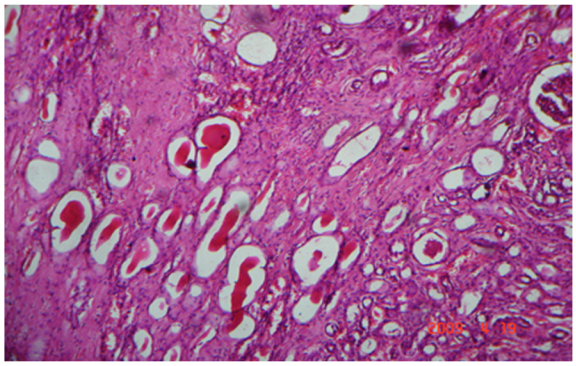
A. Chronic interstitial nephritis- thickening and fibrous tissue proliferation of interstitium, presence of inflammatory cells



B. Vascular sclerosis and dilatation of lymphatics



C. Degeneration and hyalinisation of glomeruli, loss of tubular lining epithelium



D. Presence of hyaline casts in tubules

Discussion

5. DISCUSSION

In the present study, hundred dogs with microfilariosis were studied and the results were discussed in detail.

5.1. SCREENING OF DOGS FOR MICROFILARIAE

Screening of dogs for microfilariae by wet film examination suggested that 80% of dogs with clinical signs suggestive of filariosis harbour microfilaria in their peripheral circulation. Sixteen out of eighty dogs were positive for sheathed microfilaria and remaining were nonsheathed on giemsa staining of blood smear. The prevalence of microfilariosis in dogs in Trichur was 24.2 per cent (Saseendranath *et al.,* 1986), 7.59 per cent (Radhika, 1997) and 7 per cent (Sabu *et al.,* 2005). The higher percentage of microfilariosis in the present study may be due to the effect of the population studied. The canine population utilized for screening in this study were with clinical signs suggestive of filariosis where as random screening procedures were adopted by earlier workers. The sole cause of canine microfilariosis in Kerala was identified as *Dirofilaria repens* (Radhika (1997), Suprabha and Devada (2003) and Sabu *et al.* (2005) and the microfilariae of *Dirofilaria repens* were nonsheathed. Rajendran *et al.* (1997) stated that *Wuchereria bancrofti* and *Brugia malayi* were prevalent in human beings in Thrissur district and the microfilariae of both these species were sheathed. The microfilariae of *Brugia pahangi* were sheathed and were identified as a natural parasite of dogs and cats of Africa and Far East (Kelly, 1979).

5.2. EPIDEMIOLOGY

5.2.1. Age

In the current study, microfilariosis caused by nonsheathed microfilariae was found to be more in 2 to 4 years age group (64.06 %) followed by dogs older than 4 years (28.13%) and dogs below 2 years (7.81%). The present finding was

in accordance with Radhika (1997) and Ananda and D'souza (2007). Canine dirofilariosis was more often detected in animals aged over 3 years when compared with younger dogs (Miterpakova *et al.,* 2008). The mean age of dogs with sheathed microfilariae were 2 to 4 years.

5.2.2. Breed

German Shephered (26.56%) dogs were more prone to microfilariosis due to nonsheathed microfilariae followed by Labrador (23.44%), Dachshund (17.19%), Rottweiler (10.94%), Non-descript (6.25%), Doberman, Cocker Spaniel, Boxer, Dalmatian Spitz and Great Dane. Seventy five per cent of dogs with sheathed microfilariae were belonged to Labrador breeds followed by German shephered, Rottweiler, Dobermann and Basset hound. Suprabha and Devada (2003) noticed no breed predilection in dogs infected with *Dirofilaria repens* whereas Ananda and D'souza (2007) observed highest infection rates in non descript breed followed by other breeds and the lowest observed in Labrador breeds. The higher incidence of microfilariosis in German Shephered and Labrador breeds in this study may be due to the over presentation of these breeds to the hospital.

5.2.3. Sex

High infestation rates were recorded in male dogs than females irrespective of the type of microfilariae. The higher incidence of microfilariosis in male dogs was reported by Radhika (1997) and Suprabha and Devada (2003). This might be due to the over presentation of male dogs to hospital or due to hormonal effect on susceptibility of dogs to infestation (Falls and Platt (1982), Amer (1986) and Ananda and D'souza (2007).

5.3. CLINICAL SIGNS

The clinical signs exhibited by dogs infected with nonsheathed microfilariae were anorexia, fever, vomiting, limb oedema, scrotal oedema,

conjunctivitis, cough, dyspnoea exercise intolerance, hair loss, corneal opacity, epilepsy, haemoglobinuria, ascites and nodular lesions on skin of face, neck, base of ear pinnae and tail. The clinical signs of canine dirofilariosis of the present study was in agreement with the signs observed by Jackson (1969), Tarello (2000), Atkins (2005) and Jabina and Ajith (2005). The most frequent clinical signs observed in dogs infected with sheathed microfilariae were anorexia, fever, limb and scrotal oedema, lymphangitis and conjunctivitis. According to Nutman and Weller (1998) the clinical manifestations of lymphatic filariasis in human beings may range from asymptomatic microfilariasis to hydrocele, lymphangitis, lymphadenitis with high-grade fever (filarial fever), and lymphatic obstruction. Conjunctivitis caused by adult *Brugia malayi* in a 3 year old child from Malaysia was reported by Rohela *et al.* (2006). Crandall *et al.* (1994) observed chronic lymphangitis including thrombolymphangitis and greatly dilated dermal lymphatics in oedematous limbs in ferrets inoculated with larvae of *Brugia malayi.* The gross pathologic lesions observed in golden hamsters inoculated with infective larvae of *Brugia pahangi* consisted of moderate to marked dilation of lymphatic vessels, enlargement of regional lymph nodes, and numerous lymphothrombi and emboli (Malone and Thompson, 1975).

5.4. DIAGNOSIS

5.4.1. Parasitological Investigations

5.4.1.1. Wet Film

Wet film examination revealed that 80 out of 100 dogs screened were positive for microfilariae. The four distinct patterns of motility exhibited by the microfilariae in wet blood film were wriggling, wriggling cum progressively forward, rapidly forward and sluggish forward movement across the microscopic field. Wriggling and wriggling cum progressively forward movement could be observed by Radhika (1997) on wet film examination and confirmed as *Dirofilaria repens*. *Dipetalonema reconditum* microfilariae appeared as snake

like with a rapidly forward movement across the microscopic field as observed by Hashem and Badawy (2008). Sluggish forward movement was exhibited by certain microfilariae and were later confirmed as sheathed microfilariae. Aspirate from dilated lymphatics yielded microfilaria indicating the presence of adult gravid female worms in the nearby lymphatics which may be responsible for lymphatic dilatation/lymphangitis as suggested by Basu *et al*. (2006). This is the first report of detection of microfilaria in lymphatic fluid of microfilaraemic dog.

5.4.1.2. Giemsa Staining

Giemsa stained smears of microfilaraemic dogs revealed both sheathed (20%)and nonsheathed microfilaria (80%). The microfilariae which were sheathless with a blunt head and a long tapering tail was identified as *Dirofilaria repens* (Radhika, 1997). In the nuclear column, cells did not extend up to the tail tip. Nerve ring, excretory pore and anal pore region were well appreciated in stained smears. Nonsheathed microfilariae detected on staining the vaginal discharge for exfoliative vaginal cytology were *Dirofilaria repens*. Nonsheathed microfilariae detected during cytological evaluation of nipple secretion for mammary tumour were *Dirofilaria repens* as reported by Manuali *et al.* (2005) who observed *Dirofilaria repens* microfilaria in nipple discharge of dog with a breast lump.

The nonsheathed microfilariae which were shorter with button hook like curved tail and a cephalic hook was in accordance with the morphologic characters of *Dipetalonema reconditum* as observed by Calvert and Rawlings (1993) and Ananda *et al.* (2006).

The sheathed microfilariae with pink stained sheath and two discrete overlapping nuclei at the tail end and the length of cephalic space about twice the width coincide with the description of *Brugia malayi* microfilaria made by Kanjanopas *et al.* (2001) and Kobasa *et al.* (2004).

5.4.1.3. Histochemical Staining

Histochemical staining **w**as done using commercial Acid Phosphatase Leukocyte Kit. Peribanez *et al.* (2001), Lee *et al.* (2004) and Miterpakova *et al.* (2008) utilized a commercial acid phosphatase staining kit (Leucognost–SP®, Merck, Germany) for the differentiation and species identification of canine microfilariae. Histochemical staining of nonsheathed microfilaria revealed three distinct types of microfilariae. Nonsheathed microfilaria that had acid phosphatase activity at the anal pore only is in accordance with *Dirofilaria repens* as reported by Balbo and Abate (1972) and Radhika (1997). Nonsheathed microfilariae that had acid phosphatase activity at the anal pore and central body region were also confirmed as *Dirofilaria repens* as suggested by Valcarcel *et al.* (1990) and Ananda *et al.* (2006). The staining pattern of nonsheathed microfilaria with uniform acid phosphatase activity in the body, but less intense activity cranial to the excretory pore agrees with original description of *Dipetalonema reconditum* (Chalifoux and Hunt,1971 and Toparlak *et al.*, 2005). Four out of 64 dogs with nonsheathed microfilariae were infected with *Dipetalonema reconditum*.

Histochemical staining of sheathed microfilariae revealed three distinct types based on acid phosphatase activity. Acid phosphatase staining demonstrated sheathed microfilaria with intensely positive sites at amphids, excretory and anal vesicles and phasmids and remainder of the body showed very diffuse activity and these were similar to the pattern of *Brugia malayi* observed by Kanjanopas *et al.* (2001) and Kobasa *et al.* (2004). This microfilaria was identified as *Brugia malayi* by Choochote (2008) (through e-mail). While sheathed microfilaria exhibited acid phosphatase activity at excretory and anal vesicles with very little activity in other parts were consistent with the findings of subperiodic *Brugia malayi* as described by Fan *et al*. (1985). These two patterns of enzyme activity could be observed in 15 out of 16 cases.

One dog which was imported from Russia had sheathed microfilariae, and showed intense enzyme activity uniformly throughout the body of the organism was *Brugia pahangi* which agrees with the observation made by Kanjanopas *et al.* (2001) and Kobasa *et al.* (2004).

5.4.1.4. Micrometry

The mean length and width of nonsheathed microfilariae of type 'a' and 'b' were 292 ±8.4 and 6 µm respectively. The measurements of nonsheathed microfilariae of type 'a' and 'b' were in agreement with *Dirofilaria repens* as suggested by Radhika (1997) and Ananda *et al.* (2006). Average length and width of nonsheathed microfilariae of type 'c'were 265 ± 5.6 and 5 µm respectively which was in accordance with the measurements of *Dipetalonema reconditum* as opined by Acevedo *et al.* (1981) and Soulsby (2005). The mean length of sheathed microfilariae of type '1' and '2' were 215 ± 20 and type 3 microfilariae were 280 µm respectively. The measurements of sheathed microfilariae of type '1' and '2' were consistant with *Brugia malayi* as described by Kanjanopas *et al.* (2001) and Soulsby (2005). Average length of sheathed microfilariae of type 3 were similar to that of *Brugia pahangi* as suggested by Kelly (1979).

5.4.1.5. Immunological Test

Immunological test was done using Signal MF Reagent which is a a rapid flow-through immune filtration test using recombinant filarial antigen *WbSXP-1* for detecting IgG antibodies of brugian and bancroftian filariasis in human beings in an endemic area and the sensitivity of the test was found to be 90.8% with brugian and 91.4% with bancroftian filariasis. The test showed minimum reactivity with *Loa loa* and no reactivity with *Onchocerca* or other parasitic diseases (Baskar *et al.,* 2004). Out of 16 cases with sheathed microfilariae subjected to immunospot test, 8 samples showed clear positive reaction, 4 samples showed faint reaction and the other 4 samples showed negative result. Failure of detection of antibody in certain cases might be due to

varying degrees of chronicity of the disease in these patients as reported by Baskar *et al* (2004*).* Two cases of nonsheathed microfilariae showed negative reaction indicated the absence of cross reactivity. An antibody detection dipstick test, named Brugia Rapid (BR), that detects IgG_4 antibodies reactive to a recombinant *Brugia malayi* antigen (BmR1) was developed by Rahmah *et al.* (2003) which appeared to be a promising tool for mapping and monitoring the areas endemic for brugian filariasis.

5.4.1.6. Polymerase Chain Reaction and Sequencing of Amplicon

Polymerase chain reaction technology was utilized to diagnose filarial infestations in dogs and human beings by Favia *et al.* (1996,1997), Baneth *et al.* (2002) and Mar *et al.* (2002). Results of PCR analysis using universal primer (DIDR-F1 and DIDR-R1) revealed two bands of 484 bp and 615 bp in case of mixed infection with nonsheathed and sheathed microfilariae and it corresponds to the amplification of ribosomal DNA spacer sequences of *Dirofilaria repens* and *Brugia malayi* microfilariae respectively as reported by Rishniw *et al.* (2006). Polymerase Chain Reaction analysis using *Brugia* specific (SLX 1 and SLX 2) primers revealed a band of 294 bp amplified fragment which corresponds to the amplification of SLX gene of *Brugia malayi* microfilaria coincide with the report made by Chansiri *et al.* (2002). The samples found to be positive on acid phosphatase staining were also found to be positive by PCR analysis as observed by Lee *et al.* (2004). Chansiri *et al.* (2002) utilized trans spliced leader exon 1(SLX) gene for identification of human *Brugia malayi* in domestic cats in endemic areas in Thailand and revealed that human and feline *Brugia malayi* microfilaria were identical based on PCR amplification of SLX region of these parasites.

The sequences of 484 bp amplicon when analysed using BLAST revealed 94% homology with a maximum score of 294 with the published sequence of *Dirofilaria repens* (AY693808.1) microfilaria in NCBI Gene bank data base. The sequences obtained from 294 bp PCR product when analysed using BLAST

revealed 93% homology with a maximum score of 135 with the published sequence of *Brugia malayi* (D87037.1) microfilaria in NCBI Gene bank data base. The query sequence of 10 to 101 allign with 175 to 265 of the published sequence of *Brugia malayi* (D87037.1) microfilaria. This may be due to the increasing degree of heterogenecity in the sequences of genes encoding 5S rRNA in the human parasitic nematode *Brugia malayi* as reported by Ransohoff (1989). Sequencing of the amplicon obtained with *Brugia* specific trans-spliced leader exon 1 (SLX) region primers (SLX 1 (forward) and SLX 2 (Reverse)) confirmed the diagnosis of *Brugia malayi* microfilaria. The species identification by PCR technique provided a result concordant with the microscopic and immunologic techniques. Results of micrometry, staining, immunospot test and molecular studies revealed that the newly identified parasite were similar to that of *Brugia malayi* in human beings. This is the first report of detection of *Brugia malayi* in dogs for which no previous reports were available in pubmed or other literature data bases.

5.5. CLINICAL INVESTIGATIONS

5.5.1. Electrocardiography

Electrocardiographic abnormalities associated with canine microfilariosis included increased P duration, Q wave deepening and ST coving. The mean P duration in lead II was 0.07 ± 0.11 sec. Notching of P wave was noticed in two of the cases. Duration of P wave was more than 0.04 seconds and a wide notched P wave indicated left atrial enlargement as suggested by Bolton (1975). Right ventricular enlargement was indicated by Q wave deepening. Peaking of T wave was noticed in two cases with heavy microfilaraemia and this might be due to blockage of microcirculation including the coronary vessels by large number of circulating microfilaria resulting in myocardial hypoxia and subsequent cardiac enlargement as opined by Jabina and Ajith (2005).

5.5.2. Ultrasonography

Ultrasonographic appearance of liver revealed focal hyperechoic areas to increased echogenicity of hepatic parenchyma and dilated hepatic vessels. Singh *et al.* (2007) stated that the ultrasonographic appearance of hepatic parenchyma revealed hypoechogenicity with increased visualization of many portal vein branches, which was seen as a starry sky appearance in dogs with hepatitis and diffusely hyperechoic and irregularly marginated liver could be appreciated in case of cirrhosis. Dilated hepatic veins draining into the caudal venacava were appreciated in case of hepatic congestion as evidenced by Lamb (1990).

Ultrasonogram of kidneys revealed hyperechoic areas in medullary region and renal pelvis and also echogenicity of cortex was increased with decreased cortical thickness and corresponding reduction in corticomedullary delineation. Ultrasonographic abnormalities like diffusely hyperechoic irregular shaped, small sized kidneys with poor corticomedullary delineation and decreased cortical thickness with irregular margination of capsules revealed renal parenchymal diseases in dogs as suggested by Walter *et al.* (1987). The authors also pointed out that hyperechoic kidneys were associated with renal parenchymal disorders like glomerulonephritis, glomerulosclerosis, chronic interstitial nephritis, amyloidosis, and nephrocalcinosis. The medullary rim sign demonstrated as focal hyperechoic areas in renal medullary region was suggestive of chronic interstitial nephritis and acute tubular necrosis as reported by Biller *et al.* (1992). According to Mantis and Lamb (2000) dogs with renal disease and medullary rim sign are likely to have other ultrasonographic signs of renal disease whereas finding medullary rim sign alone is more likely in dogs without renal dysfunction.

5.5.3. Radiography

Radiographs of four dogs with limping, painful lymphangitis and unilateral limb oedema revealed no orthopaedic abnormalities and only soft tissue swelling was seen. (Biery, 1985).

5.6. CLINICAL PATHOLOGY

5.6.1. Haematobiochemical Analysis

5.6.1.1. Nonsheathed Microfilariae

5.6.1.1.1. Haemogram

In all the treatment groups, a non significant reduction in total erythrocyte count was noticed when compared with healthy controls as observed by Sharma and Pachauri (1982) and Ananda and D'souza (2006) in dogs infected with *Dirofilaria repens.* Anaemia in canine filariosis might be due to haemolysis as a result of destructive motility of microfilariae as suggested by Kitagawa *et al.* (1989). In all the groups, the post treatment values of total erythrocyte count, haemoglobin and volume of packed red cells were increased, but not significantly different from the pre treatment values.

The mean values of erythrocyte sedimentation rate in dogs of group I, II, III, IV and V were 8.83 ± 4.38, 3.83 ± 0.60 , 4.67 ± 1.10 , 4.83 ±1.49 and 7.83 ± 2.83 mm/hr respectively. Statistically significant increase was observed in ESR value on day 1 in animals of all the five groups when compared to normal value and this might be due to anaemia or chronic pathology of the disease or auto agglutination of red blood cells observed in this disease during infection (Hashem and Badawy, 2008). Increase in ESR could also be associated with the extensive tissue damage as occurred in this condition. Similar observations were made by Sharma and Pachauri (1982) in canine dirofilariasis. A regenerative anaemia of macrocytic hypochromic type along with a reduction in the RBC count with an increase in ESR was observed by Hashem and Badawy (2008) with

Dipetalonema reconditum infestation in dogs. The values decreased significantly and returned to normal level in animals of group I, II and V on day 7.

5.6.1.1.2. Leucogram

A statistically significant leukocytosis with non significant neutropenia was observed on day 1 in animals of group I, II , III, IV and V when compared to normal value. These findings were in agreement with those of Sharma and Pachauri (1982), Ananda and D'souza (2006). In contrary to this a neutrophilic leukocytosis was observed in microfilaraemic dogs by Paltrinieri *et al.* (1998), Niwetpathomwat *et al.* (2007) and Hashem and Badawy (2008) and this might be due to increased phagocytic removal of tissue breakdown products of microfilariae or inflammatory response to the parasite. The reduction in post treatment total leucocyte count in all the groups were significant and the value became comparable to normal control.

Non significant lymphocytosis with a significant eosinophilia was observed on day 1 in animals of all the five groups. Similar findings were made by Sharma and Pachauri (1982) and Ananda and D'souza (2006) while Wittwer *et al.* (1979) found no significant change in differential leukocyte count in dogs infested with microfilaria of *Dipetalonema reconditum*. Lymphocytosis in microfilaraemic dogs was due to intense antigenic stimulations which increase the demands for lymphocytes to be transformed into plasma cells for production of antibodies as observed by Hashem and Badawy (2008). Eosinophilia might be due to sensitivity to the foreign protein of a parasite which may be a part of an immune phenomenon as opined by Feldman *et al.* (2000). The reduction in eosinophil count of animals of all groups on day 7 were statistically significant when compared to its pre treatment values.

5.6.1.1.3. Platelet Count

Statistically significant thrombocytopenia was noticed on day 1 in animals of all treatment groups and the post treatment values showed a statistically

significant increase and the values were comparable to normal value. The thrombocytopenia in microfilaraemic animals might be due to immune mediated platelet destruction. This is in accordance with the findings of Warner *et al.* (1995), Anuchai *et al.* (2006) and Niwetpathomwat *et al.* (2007) who observed thrombocytopenia in dogs infected with *Dirofilaria immitis.* Ananda and D'souza (2006) also observed a marked reduction in platelet count (191.20 ± 15.14 thousands/mm^3) in *Dirofilaria repens* microfilaraemic dogs when compared to normal dogs.

5.6.1.1.4. Serum Total Protein, Albumin, Globulin and AG Ratio

Statistically significant hyperproteinemia and hyperglobulinaemia with non significant reduction in AG ratio could be observed in animals of group II and IV on day1 when compared to healthy control group while non significant increase in serum total protein level with hyperglobulinaemia and reduction in AG ratio were observed in animals of group I, III and V. Hyperproteinaemia observed in the present study might be due to increase in the globulin concentration in response to the parasitic antigens or due to release of haemoglobin from lysed erythrocyte as suggested by Moustafa *et al*. (1991). Non significant reduction in AG ratio corresponds to the degenerative changes in the liver. Kitagawa *et al*. (1998) observed hypoalbuminemia in dogs with heartworm disease and opined that the hypoalbuminemia corresponds to the degenerative changes in haemoparasitized organs mainly liver. Urinary loss of proteins resulted in hypoalbuminaemia (Atkins, 2005). Hyperproteinemia (8.95 ± 0.16 g/dl), hypoalbuminemia (3 ± 0.04 g/dl) and hyperglobulinemia (5.95 ± 0.32 g/dl) with a reduced albumin globulin ratio were reported by Hashem and Badawy (2008) in *Dipetalonema reconditum* infected dogs.

5.6.1.1.5. Serum ALT, AST and ALP

The pre treatment serum ALT, AST and ALP values in all the groups showed a statistically significant increase when compared to healthy control.

This was in confirmity with the results obtained by Calvert and Rawlings (1993), Ananda and D'souza (2006) Niwetpathomwat *et al.* (2007) in microfilaraemic dogs. The increased serum enzymes demonstrated in microfilaraemic dogs revealed liver dysfunction secondary to circulatory disturbance (Hashem and Badawy, 2008) or due to localization of a large number of circulating microfilaria in the hepatic portal vein (Ananda and D'souza, 2006). All these enzymes are predominantly intracellular and so low serum enzyme activity in healthy animals and any increase in serum activity would be a reflection of damage to the tissue in which they are lodged (Niwetpathomwat *et al.*, 2007). A significant decrease in serum ALT and ALP level was observed on day 7 in all groups except group IV when compared to corresponding pre treatment values, but did not return to normal level. But the reduction in AST level was statistically non significant in all the groups on day 7 when compared to day 1 and was not comparable to healthy controls. Maximum post treatment reduction in serum ALT occurred in group II while serum AST and ALP showed similar patterns in all the five groups. Even after seven days of treatment the serum enzymes did not come down to normal level. This implies that therapeutic approach towards canine microfilariosis may require additional supportive therapy to improve hepatic function along with specific therapy.

5.6.1.1.6. Blood Urea Nitrogen and Creatinine

Animals of all the groups showed a statistically significant increase in BUN and creatinine values on day 1 when compared to healthy controls. These findings were in agreement with Sharma and Pachauri (1982), Ananda and D'souza (2006), Anuchai *et al.* (2006), Niwetpathomwat *et al.* (2007) and Hashem and Badawy (2008) who observed significantly higher blood urea nitrogen and serum creatinine in canine microfilariosis. This might be due to severe kidney dysfunction and intravascular haemolysis associated with the infection as opined by Kitagawa *et al*. (1989) and Anuchai *et al.* (2006). The corresponding post treatment values in group I, II, III and V showed a significant decrease when compared with values on day1 and the values did not return to

normal level. The creatinine values of animals of all groups still remained significantly higher than healthy controls. Immune mediated glomerular nephritis, glomerulo sclerosis (Grauer *et al*, 1989) chronic interstitial nephritis and amyloidosis (Rawlings, 1986) were observed in dogs infected with *Dirofilaria immitis* and this might have contributed to this elevated BUN and serum creatinine. Maximum post treatment reduction in BUN and creatinine observed in group I and V respectively.

5.6.1.2. Sheathed Microfilariae

5.6.1.2.1. Haemogram, Leucogram and Platelets

Haematobiochemical studies of dogs affected with sheathed microfilariae (group VI) revealed mild anaemia with severe leucocytosis, neutropenia, lymphocytosis, eosinophilia and severe thrombocytopaenia. Statistically significant leucocytosis with non significant neutropenia and lymphocytosis were observed on day 1 in comparison with healthy controls. Eosinophilia and erythrocyte sedimentation rate observed in animals of group VI on day 1 were highly significant when compared to normal value. Similar haematological changes were observed in dogs affected with non sheathed microfilariae. Spry *et al.* (1981) assessed patients with tropical filarial eosinophilia and suggested that blood eosinophils were induced to release their granule constituents into the circulation which might interact with microfilariae, and led to some of the clinical features of chronic filarial infections. The mean values of total leucocyte count, eosinophils, ESR and platelets were reduced significantly on day 7 when compared to corresponding pre treatment values.

5.6.1.2.2. Serum Biochemical Analysis

Significantly increased activities of serum enzymes, ALT, AST and ALP with hyperproteinemia, hyperglobulinaemia and non significant reduction in AG ratio were observed and it might be attributed to liver damage as observed in case of dogs with nonsheathed microfilariae. Statistically significant reduction in total

protein and globulin with non significant increase in A:G ratio could be observed on day 7 when compared to day 1. The significantly higher blood urea nitrogen and creatinine on day 1 of animals of group VI suggested kidney dysfunction associated with the condition. Significant reduction in creatinine with a non significant reduction in BUN could be observed on day 7 when compared to day 1 and was not comparable to normal value.

5.6.2. Urinalysis

5.6.2.1. Qualitative Analysis

Qualitative urinalysis using dipstick revealed the presence of urine protein (4+), blood pigments and bile pigments with a mean specific gravity of 1.010. Similar observations were made by Atkins *et al.* (2000) in cats with dirofilariasis and Forterre *et al.* (2004) and Raila *et al.* (2007) in dogs with chronic renal disease. Proteinuria with reduced specific gravity suggested more severe renal involvement of the condition. Microalbuminuria was an important indicator of early renal damage (Langston, 2004) and was always associated with underlying systemic disease (Whittemore *et al.,* 2006). Forterre *et al.* (2004) suggested that measurement of total urinary proteins in dogs by urinary dipstick was a typical method for assessing the presence of potentially serious renal disorders. The presence of kidney disease was indicated by a significantly elevated proteinuria in humans with lymphatic filariasis from *Brugia malayi* endemic areas of Kerala and patients with filarial fever showed purely tubular disorders, but in other cases with lymphangitis alone exhibited a mixed type of proteinuria (Langharnrner *et al.,* 1997). Raila *et al.* (2007) stated that urinalysis in a 14-month-old bernese mountain dog with chronic renal failure revealed a specific gravity of 1.020 and an overt proteinuria with the abnormal urine protein creatinine ratio (UPC) of 2.50.

5.6.2.2. Quantitative Analysis

The urinalysis of microfilaraemic dogs revealed significant increase in NAG (17.54 ± 1.65 U/g of creatinine), UPC (1.91 ± 0.67), γ GT (1.81 ± 0.59IU/L) and ALP (15.79 ± 8.40 IU/L) when compared to nonmicrofilaraemic dogs. N-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyl transpeptidase (GGT) are renal tubular enzymes which are primarily located in the lysosomes and brush border respectively of the proximal convoluted tubule and these enzymes were released into the urine as a result of renal tubular injury (Clemo, 1998). Increases in urine NAG and GGT indices allow for earlier detection of renal tubular damage in dogs as reported by Brunker *et al.* (2009). An increased urine protein creatinine ratio of 1.62 was observed by Forterre *et al.* (2004) in dogs with chronic renal disease. The measurement of activity of renal tubular enzymes like NAG, GGT and alkaline phosphatase were more sensitive for detection of acute renal damage than the current standard veterinary diagnostic tests like assessment of serum creatinine, blood urea nitrogen (BUN) and urine specific gravity (Cowgill and Francey, 2005). Increased urinary NAG activity was observed by Sato *et al.* (2002) in dogs with chronic renal failure and Langharnrner *et al.* (1997) in human brugian filariasis patients (1.4 ± 0.64 U/mM/C) from Kerala.

The elevated levels of serum total protein, globulin, serum enzymes like ALT and ALP and nonsignificant reduction in AG ratio suggestive of liver pathology in microfilaraemic dogs. Elevated levels of BUN, creatinine, urine protein creatinine ratio, NAG, ALP, proteinuria with low specific gravity confirmed the renal involvement in microfilaraemic dogs irrespective of the type of microfilaria involved in the disease process. This multiorgan pathology in canine microfilariosis suggested the involvement of toxic and immunological effects of these parasite in the pathogenesis of the disease as suggested by Kamalu (1991).

5.7. TREATMENT

5.7.1. Clinical Response

5.7.1.1. Nonsheathed Microfilariae

Clinical response was evaluated by the periodic clearance of microfilariae on wet blood film examination. In animals of treatment group I (treated with single oral dose of ivermectin @ 50µg/kg body weight), the percentage efficacy of the drug in clearing microfilaria is 50, 70 and 100 in 2nd (24 hours), 3rd (48 hours) and 4th (72 hours) day of treatment respectively. Eventhough complete clearance was observed in 72 hours post treatment, rapid reduction in microfilarial counts were observed within 24 hours. Similar findings were observed by Clavert and Rawlings (1993). In contrary to this, the efficacy of single oral dose of ivermectin @ 50µg/kg body weight to clear microfilaraemia was 100% within 24 hours (Blair *et al.*, 1983) and 90% in 3 weeks (Neer and Hoskins, 1989).

In case of treatment group II, (treated with single oral dose of ivermectin @ 100 µg/kg body weight) the rate of clearance of microfilaraemia was 50 and 100% on 2nd (24 hours) and 3rd (48 hours) day of treatment trial respectively. Maximum microfilaricidal efficacy of ivermectin was attained @ 100 µg/kg body weight. Soulsby (2005) reported that an oral administration of ivermectin @ 0.05-0.1 mg/kg body weight reduced microfilaremia by 90% within 24 hrs

In animals of group III (treated with single oral dose of ivermectin @ 200 µg/kg body weight), 30, 70 and 100% clearance of microfilaraemia were attained by day 2, 3 and 4 post treatment respectively. Complete clearance of microfilaraemia was attained in group I and III by day 4 and group II by day 3. This indicated the non dose dependency of ivermectin. Similar findings were reported by Blair *et al.* (1983) who observed almost similar efficacy for single oral dose of ivermectin @ 0.2 mg/kg, 0.05mg/kg and 0.0125 mg/kg in eliminating all the microilariae within 24 hours. Radhika *et al.* (1999) opined

that single subcutaneous injection of ivermectin @ 200 and 333 µg/kg body weight were equally effective against *Dirofilaria repens* microfilariae.

No periodic clearance of circulating microfilaria could be observed in dogs of group IV (treated with levamisole hydrochloride @ 5 mg/kg body weight orally for 7 days) even after seven days of treatment. Similar results were observed by several researchers. Bradley (1976) evaluated the microfilaricidal efficacy of orally administered levamisole resinate @ 5.5 mg/kg body weight/day for 10 days in dogs infected naturally or experimentally with *Dirofilaria immitis* and suggested that complete clearance was not obtained even after 10 days of treatment. Bradley and Alford (1977) found 100% efficacy within 4 weeks with levamisole given @ 4.4 mg/kg body weight twice daily for 2 weeks, followed by 6.6 mg/kg body weight twice daily for 2 weeks.

The percentage clearance rate in animals of group V (treated with levamisole hydrochloride @ 10 mg/kg body weight orally for 7 days) were 20, 50, 80 and 100 % on day 2, 3, 4 and 7 respectively. Complete clearance of microfilaraemia was attained in group V by day 7 of treatment trials. Levamisole resinate @ 11mg/kg body weight was effective in reducing the microfilaria counts to zero within 10 days (Bradley, 1976). Carlisle *et al.* (1984) found 100% efficacy with levamisole hydrochloride @ 10 mg/kg body weight twice daily for 14 days against the microfilaria of *Dirofilaria immitis* in dogs, and suggested that a dose rate of 20 mg/kg body weight was effective, but toxic. The administration of levamisole hydrochloride @ 7.5mg/kg body weight subcutaneously for seven consecutive days (Radhika *et al.*,1999) and @ 10-12 mg/kg body weight orally for 30 days (Dillon, 2000) were found to be effective in canine microfilariosis.

5.7.1.2. Sheathed Microfilariae

In animals of group VI, treatment trials were conducted with single oral dose of ivermectin @ 50, 100 and 200 µg/kg body weight and levamisole hydrochloride @ 5 and 10 mg/kg body weight orally for 7 days. Single oral dose

of ivermectin at all the above doses were not found to be effective in animals of group VI. Phantana *et al.* (2002) investigated the efficacy of treating cats, naturally infected with *Brugia malayi*, with a single dose of ivermectin (0.05 and 0.1 µg/kg orally and 0.2, 0.4 and 1 mg/kg injected subcutaneously) and the results indicated that the microfilarial density of all ivermectin-treated cats significantly dropped after 30 and 60 days of treatment. In cats receiving 0.2 mg/kg, the number of microfilariae decreased by 75–90% at day 30 after drug treatment and further reduced to nearly 100% on day 60 after receiving a repeat dose given at day 30.

All the animals responded well to the treatment with levamisole hydrochloride @ 10 mg/kg body weight orally for 7 days. The periodic clearance rate of microfilaraemia in animals of group VI were 18.75, 43.75 and 100% on 2^{nd}, 3^{rd} and 4^{th} day of treatment respectively. Treatment of *Brugia malayi* microfilaraemic patients with a combination of mebendazole 500 mg thrice daily for 21 days and levamisole hydrochloride 2.5 mg/kg weekly for 3 weeks was effective in hastening amicrofilaraemia within second post treatment week (Mak and Chan, 1983). No reports are traceable to interpret the efficacy of levamisole hydrochloride @ 10 mg/kg body weight as far as sheathed microfilaria is concerned. The current antifilarial therapies in human beings (infected with sheathed microfilariae) were restricted to diethyl carbamazine or ivermectin in combination with albendazole (Ottesen *et al.,* 1997).

5.7.2. Treatment Response

5.7.2.1. Nonsheathed Microfilariae

Animals of all the groups except group IV responded very well to the concerned treatment. Remission of clinical signs was observed in all the animals treated in group I, II, III and V. No recurrence was reported so far in dogs received treatment with single oral dosage of ivermectin @ 50, 100 and 200µg/kg body weight and this might be due to the partial adulticidal properties of

ivermectin (McCall *et al.,* 1998) or due to the suppressive effect of ivermectin on microfilaraemia as opined by Lok *et al.*(1988) who observed that the effects of ivermectin on embryogenesis of microfilariae within female heart worm indicated that not only the early developmental stages but also the latter developmental stages into the circulation were interrupted. But recurrence was reported in one case of group V one month after treatment and this might be due to the presence of adult worms. Clinical signs and microfilaraemia persisted as such in eight animals of group IV even after 7 days of treatment as observed by Bradley (1976) and two animal of this group died during the course of therapy.

5.7.2.2. Sheathed Microfilariae

All the animals of group VI were successfully treated with levamisole hydrochloride @ 10 mg/kg body weight for seven days. No recurrence was reported in animals of group VI there after. Remission of almost all clinical signs except lymphangitis were observed. In one animal limb oedema was reduced much, but still persisted even after 7 days of treatment. Lymphangitis persisted in all the 5 animals even after the animal became amicrofilaraemic resulting in permanent disability. Similar findings were observed in human beings by Dasgupta *et al.* (1984), Nutman and Weller (1998) and Ottesen (2006).

5.7.3. Adverse Drug Reactions

Erratic drug reactions with ivermectin @ 200µg/kg body weight and levamisole hydrochloride @ 10 mg/kg body weight orally for seven days were observed in microfilaraemic animals (Page, 2002).

Clinical response could be observed on day 7 in animals of all the groups except group IV. The post treatment values of haematology revealed that all the treatments were equally effective to normalize the haematological alterations. The post treatment reduction in serum ALT was maximum in group II while serum AST and ALP showed similar patterns in all the five treatment groups. Maximum post treatment reduction in BUN and creatinine happened in animals

of group I and V respectively. Thus single oral dose of ivermectin @ 100 µg/kg body weight and levamisole hydrochloride @ 10 mg/kg body weight orally for seven days were found to be most effective for canine microfilariosis. Complete clearance of microfilariae on wet blood film could be observed within 48 hours in animals treated with single oral dose of ivermectin @ 100 µg/kg body weight when compared to other groups and no adverse drug reactions observed in such animals. The treatment cost for an average dog weighing 30 kg with ivermectin @ 100µg/kg body weight is Rs 3.25/- while the treatment cost with levamisole hydrochloride @ 10 mg/kg body weight orally for seven days is Rs 277.20/-. Considering all these aspects, single oral dose of ivermectin @ 100 µg/kg body weight can be selected as a suitable treatment modality for microfilariosis in dogs. Levamisole hydrochloride @ 10 mg/kg body weight for seven days was the only effective treatment for microfilariosis due to *Brugia malayi* in dogs under the present study.

Result of post treatment values of biochemical parameters revealed that many of the parameters like ALT, ALP and BUN were still in elevated level. Based on above studies it concluded that follow-up evaluation of these parameters could be a relevant approach to find out the therapeutic effectiveness. A therapeutic plan should consists of both specific and clinically supportive treatments to imporove hepatic and renal function, to achieve maximum goal.

5.8. AUTOPSY AND HISTOPATHOLOGY

The gross pathological lesions observed with microfilariosis were fibrosis and congestion of liver, congestion of lungs and alveolar emphysema, cardiac hypertrophy and small and pale kidneys with granular pitted surface. These findings were consistant with the lesions observed in animals infected with *Dirofilaria immitis* (Otto and Jackson, 1969) and *Dirofilaria repens* (Kamalu, 1991) and (Dzaja *et al.*, 2008). An adult worm retrived from the subcutaneous tissue was about 15cm long with striated cuticle and was identified as *Dirofilaria repens* (Sabu *et al.*, 2005).

The hispathological examination revealed lesions in heart, lungs, liver and kidneys in microfilariosis. Heart with myofibrillar fragmentation and lungs with congestion of capillaries and atlectasis, and thromboemboli formation and liver with passive congestion, cirrhosis and portal hepatitis were noticed. Similar pathological lesions were observed by Otto and Jackson (1969), Rawlings (1986) and (Dzaja *et al.*, 2008). The kidneys revealed vacuolation, necrosis and predominantly hyalinization and atrophy of glomeruli with the presence of dense granular deposits adjacent to the glomeruli, diffuse tubular necrosis with desquamation of tubular epithelium and fibrous tissue proliferation in the interstitium indicating chronic interstitial nephritis. This is consistant with the findings of Kamalu (1991) in dogs infected with *Dirofilaria repens* and Shirota *et al.* (1979) in *Dirofilaria immitis* infected dogs. The renal tubules with hyaline cast indicating the presence of proteinuria. Persistant proteinuria, being a marker of renal disease was associated with progressive glomerular and tubulointerstitial lesions resulting in loss of nephrons as observed by Grauer (2005) in dirofilariasis. Paes-de-Almeida *et al.* (2003) suggested that the pathogenesis of kidney disease in dirofilariasis was associated with deposition of immune complexes in the glomerular basement membrane resulting in thickening of the glomerular basement membrane and the presence of dense deposits in the glomerular basement membrane. Sutton (1988) described immune mediated glomerulonephropathy in dogs infested with *Dirofilaria immitis* while Grauer *et al* (1989) observed glomerulosclerosis in experimentally induced *Dirofilaria immitis* in dogs. Chronic interstitial nephritis was observed with sheathed microfilariae in dogs as observed with renal abnormalities in chronic filariasis in humans by Neilson (1989). Histopathological findings observed in the present study were well correlated with biochemical and ultrasonographic findings.

Summary

6. SUMMARY

The present study entitled "Clinico-therapeutic studies on canine microfilariosis" was conducted in the Department of Clinical Veterinary Medicine, College of Veterinary and Animal Sciences, Mannuthy during the period of 2007-2009.

Dogs presented to Veterinary College Hospital, Mannuthy and University Veterinary Hospital, Kokkala from different parts of Kerala were utilized for the study. Hundred dogs of both sexes belonging to various breeds and above 6 months of age presented with clinical signs suggestive of microfilariosis like fever, anorexia, vomiting, conjunctivitis, lymphangitis, limb and scrotal oedema were screened for microfilaria by wet film examination. Wet film examination revealed that 80% of dogs were positive for microfilaria.Staining of blood smear with giemsa (1:10) demonstrated that 16 out of 80 dogs were positive for sheathed microfilaria and remaining were nonsheathed. Out of these 50 (nonsheathed) microfilaremic dogs were selected and treated at random with five schedules of treatment so that each schedule consisted of ten animals each (Group I, II, III, IV and V). Sheathed microfilaraemic animals were considered as a separate group for treatment trial. All these animals were subjected to periodic wet film examination on 2^{nd}, 4^{th} and 7^{th} day of treatment to assess the treatment response.

Animals of different groups will be treated as follows:

Group I- Single oral administration of ivermectin @50 µg/kg body weight.

Group II- Single oral administration of ivermectin @100 µg/kg body weight.

Group III - Single oral administration of ivermectin @200 µg/kg body weight.

Group IV- Oral administration of levamisole hydrochloride @ 5mg/kg body weight daily for 7 days.

Group V- Oral administration of levamisole hydrochloride @ 10mg/kg body weight daily for 7 days.

Group VI - Oral administration of levamisole hydrochloride @ 10 mg/kg body weight daily for 7 days since initial treatment trial as in the case of group I,II,III and IV were not satisfactory.

High infestation rates were recorded in male dogs of 2-4 years of age than females irrespective of the type of microfilariae. High incidence of microfilariosis with non sheathed microfilariae was observed in GSD followed by Labrador, Dachshund, Rottweiler etc and with sheathed microfilariae in Labrador breeds followed by German shephered, Rottweiler etc.

Diagnosis was made by parasitological studies, immunological and molecular techniques, clinical investigations and haematobiochemical analysis.

Parasitological studies consisted of wet film examination, giemsa staining and histochemical staining. The patterns of motility exhibited by the *Dirofilaria repens* microfilariae in wet blood film were wriggling and wriggling cum progressively forward movement across the microscopic field while *Dipetalonema reconditum* microfilariae appeared as snake like with a rapidly forward movement across the microscopic field. On giemsa staining, the microfilariae which were sheathless with a blunt head and a long tapering tail was identified as *Dirofilaria repens.* The microfilariae which were sheathless, shorter with button hook like curved tail shaped and a cephalic hook was *Dipetalonema reconditum.* The sheathed microfilariae with pink stained sheath and two discrete overlapping nuclei at the tail end and the length of cephalic space about twice the width coincide with the description of *Brugia malayi* microfilariae.

Histochemical staining **w**as done using commercial Acid Phosphatase Leukocyte Kit. Histochemical staining of nonsheathed microfilaria revealed three distinct types: a) microfilaria in which the enzyme activity demonstrated at the anal pore only. b) microfilaria in which the enzyme activity demonstrated at the

anal pore and central body region (diffuse). c) microfilaria exhibiting uniform enzyme activity, but less intense activity cranial to the excretory pore. The first two patterns were shown by *Dirofilaria repens* microfilariae and the third by *Dipetalonema reconditum.*

Histochemical staining of sheathed microfilaria also revealed three distinct types: 1) microfilaria in which enzyme activity restricted to amphids, excretory and anal vesicles and phasmids. 2) microfilaria in which enzyme activity restricted to excretory and anal vesicles. 3) microfilaria exhibiting intense enzyme activity uniformly throughout the body of the organism. The first two patterns were shown by *Brugia malayi* microfilariae and the third by *Brugia pahangi.*

Micrometric measurements of identified parasites were consistant with earlier findings of *Dirofilaria repens* , *Dipetalonema reconditum* , *Brugia malayi* and *Brugia pahangi.*

Out of 16 cases with sheathed microfilariae subjected to immunospot test, 8 samples showed clear positive reaction, 4 samples showed faint reaction and the other 4 samples showed negative result.PCR analysis using universal primer (DIDR-F1 and DIDR-R1) revealed two bands of 484bp and 615bp in case of mixed infection which corresponds to amplification of ITS-2 region of *Dirofilaria repens* and *Brugia malayi* microfilariae respectively. Polymerase Chain Reaction (PCR) analysis using *Brugia* specific (SLX 1 and SLX 2) primers revealed a band of 294 bp PCR fragment which corresponds to the amplification of SLX gene of *Brugia malayi* microfilaria.

The sequence obtained on sequencing of 294 bp and 484 bp amplicon when analysed using BLAST revealed 93 and 94% homology with the published *Brugia malayi* and *Dirofilaria repens* gene partial sequence respectively and were subsequently submitted to the Genbank data base at the National Centre for

Biotechnology Information and assigned accession numbers (FJ717408 and FJ717410).

Results of micrometry, staining techniques, immunospot test and molecular studies revealed that the newly identified parasite were similar to that of *Brugia malayi* in human beings. This is the first report of detection of *Brugia malayi* in dogs for which no previous reports were available in pubmed or other literature data bases. Also this is the first report of *Brugia pahangi* in a dog from India and *Dipetalonema reconditum* from dogs of Kerala.

Clinical investigations included ECG, ultrasonography and radiography. Electrocardiographic abnormalities associated with microfilaraemic dogs were increased P duration, Q wave deepening, ST coving, notching of P wave and peaking of T wave.Major changes observed in ultrasonogram of liver were focal hyperechoic areas to increased echogenicity of hepatic parenchyma and dilated hepatic vessels. The echogenicity of cortex was increased with decreased cortical thickness and corresponding reduction in corticomedullary delineation. Radiographs of four dogs with limping, painful lymphangitis and unilateral limb oedema were taken to rule out orthopaedic problems if any. Plain lateral radiographs of metacarpal region revealed no break in the continuity of bone indicating absence of orthopaedic abnormalities.

Haematobiochemical studies of dogs affected with both non sheathed and sheathed microfilariae revealed mild anaemia with severe leucocytosis, neutropenia, lymphocytosis, eosinophilia, elevated ESR and severe thrombocytopaenia. Hyperproteinemia with hyperglobulinaemia and non significant reduction in AG ratio could be observed in such animals. Statistically significant increase in serum ALT, AST, ALP, BUN and creatinine values was also observed when compared to healthy controls.

Qualitative urinalysis revealed the presence of urine protein (4+), bilirubin, blood pigments and bile pigments with a mean specific gravity of

1.010. The urinalysis of microfilaraemic dogs revealed significant increase in NAG (17.54±1.65 U/g of creatinine), UPC (1.91±0.67), γ GT (1.81±0.59IU/L) and ALP (15.79±8.40 IU/L) when compared to nonmicrofilaraemic dogs.

The elevated levels of serum total protein, globulin, serum enzymes like ALT and ALP and nonsignificant reduction in AG ratio suggestive of liver pathology in microfilaraemic dogs. Elevated levels of BUN, creatinine, urine protein creatinine ratio, NAG, ALP, proteinuria with low specific gravity confirmed the renal involvement in microfilaraemic dogs irrespective of the type of microfilaria involved in the disease process. This multiorgan pathology in canine microfilariosis suggested the involvement of toxic and immunological effects of these parasite in the pathogenesis of the disease.

The clinical response was evaluated by the periodic clearance of microfilariae on wet blood film examination. In group I, 50% of animals cleared off microfilariae by day 2 and 70% on day 3 and 100% on day 4. In group II, 50% each of animals cleared off microfilariae on day 2 and day 3. Thirty percent dogs were negative for microfilaraemia on day 2 and 70% on day3 and complete clearance by day 4 in group III. No periodic clearance of circulating microfilaria could be observed in dogs of group IV even after seven days of treatment.The percentage clearance rate in animals of group V were 20, 50, 80 and 100 % 0n day 2, 3, 4 and 7 respectively. Erratic drug reactions with ivermectin @ 200µg/kg body weight and levamisole hydrochloride @ 10 mg/kg body weight orally for seven days were observed in microfilaraemic animals.

All the animals of group VI were successfully treated with levamisole hydrochloride @ 10 mg/kg body weight for seven days since initial treatment as in case of group I, II, III and IV were not satisfactory. Lymphangitis persists in all the 5 animals even after the animal become amicrofilaraemic. In group VI, 18.75 % of dogs were cleared off microfilaraemia by day 2 followed by 43.75 on 3rd and 100% on 4th day of treatment respectively.

Clinical response could be observed on day 7 in animals of all the groups except group IV. The post treatment values of haematology revealed that all the treatments were equally effective to normalize the haematological alterations. The post treatment reduction in serum ALT was maximum in group II while serum AST and ALP showed similar patterns in all the five treatment groups. Maximum post treatment reduction in BUN and creatinine happened in animals of group I and V respectively. Thus single oral dose of ivermectin @ 100 µg/kg body weight and levamisole hydrochloride @ 10 mg/kg body weight orally for seven days were found most effective for canine microfilariosis. Complete clearance of microfilariae on wet blood film could be observed within 48 hours in animals treated with single oral dose of ivermectin @ 100 µg/kg body weight when compared to other groups and no adverse drug reactions observed in such animals and the treatment is economical too. Considering all these aspects, single oral dose of ivermectin @ 100 µg/kg body weight can be selected as a treatment modality for microfilariosis due to *Dirofilaria repens* and *Dipetalonema reconditum* in dogs. Levamisole hydrochloride @ 10 mg/kg body weight for seven days was the only effective treatment for microfilariosis due to *Brugia malayi* in dogs.

Two animals died during the course of treatment were subjected to post mortem examination. The gross pathological lesions observed with microfilariosis were fibrosis and congestion of liver, congestion of lungs and alveolar emphysema, cardiac hypertrophy and small and pale kidneys with granular pitted surface. An adult worm retrived from the subcutaneous tissue was about 15cm long with striated cuticle and was identified as *Dirofilaria repens.* The hispathological examination revealed lesions in heart, lungs, liver and kidneys in microfilariosis. Heart with myofibrillar fragmentation and lungs with congestion of capillaries and atlectasis ,and thromboemboli formation and liver with passive congestion and portal hepatitis were noticed. The kidneys revealed vacuolation, necrosis and predominantly hyalinization and atrophy of glomeruli with the presence of dense granular deposits adjacent to the glomeruli, diffuse

tubular necrosis with desquamation of tubular epithelium and fibrous tissue proliferation in the interstitium indicating chronic interstitial nephritis. The renal tubules with hyaline cast indicating the presence of proteinuria.

Result of post treatment values of hepatic and renal function test revealed that many of the parameters like ALT, ALP and BUN were still in elevated level. Based on above studies it concluded that follow- up evaluation of these parameters could be a relevant approach to find out the therapeutic effectiveness. A therapeutic plan should consists of both specific and clinically supportive treatments to improve hepatic and renal function.

FUTURE PLAN OF WORK

1. To develop a suitable prophylactic and therapeutic approach to control and treat canine microfilariosis in this increasing ewe of chronic renal diseases in microfilaraemic dogs.

2. Elucidate the possible role of dogs in the transmission of human filariasis.

3. Suitable therapeutic approach to be developed to treat canine microfilariosis due to *Brugia malayi* which will be helpful to decrease the risk to humans in the vicinity of the infected animals when suitable mosquito vectors are present.

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CLINICO-THERAPEUTIC STUDIES ON CANINE MICROFILARIOSIS

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ABSTRACT

A study on canine microfilariosis was conducted in the Department of Clinical Veterinary Medicine, College of Veterinary and Animal Sciences, Mannuthy during the period of 2007-2009. Hundred dogs of both sexes belonging to various breeds and above 6 months of age presented to Veterinary College Hospital, Mannuthy and University Veterinary Hospital, Kokkala from different parts of Kerala with clinical signs suggestive of microfilariosis were screened for microfilaria by wet film examination. Wet film examination revealed that 80% of dogs were positive for microfilaria. Staining of blood smear with giemsa (1:10) demonstrated that 16 out of 80 dogs were positive for sheathed microfilaria and remaining were nonsheathed. Out of these 50 (nonsheathed) microfilaremic dogs were selected and treated at random with five schedules of treatment so that each schedule consisted of ten animals each (Group I, II, III, IV and V). Sheathed microfilaraemic animals were considered as a separate group for treatment trial. All these animals were subjected to periodic wet film examination on 2^{nd}, 4^{th} and 7^{th} day of treatment to assess the treatment response.

High infestation rates were recorded in male dogs of 2-4 years of age than females irrespective of the type of microfilariae. High incidence of microfilariosis with non sheathed and sheathed microfilariae was observed in GSD and Labrador breeds respectively.

Diagnosis was made by parasitological studies, immunological and molecular techniques, clinical investigations and haematobiochemical analysis.

In wet film examination distinct patterns of motility was observed with the type of microfilariae present. The speciation of microfilariae were done based on morphological characteristics in giemsa stained smears, acid phosphatase enzyme activity, immunospot test and PCR analysis and sequencing of amplicon. The different species of microfilariae identified were that of *Dirofilaria repens,*

Dipetalonema reconditum, Brugia malayi and *Brugia pahangi.* Of which *Brugia malayi* and *Brugia pahangi* were sheathed. Results of micrometry, staining, immunospot test and molecular studies revealed that the newly identified parasite were similar to that of *Brugia malayi* in human beings. This is the first report of detection of *Brugia malayi* in dogs for which no previous reports were available in pubmed or other literature data bases. Infact this is also the first report of *Brugia pahangi* in a dog from India and *Dipetalonema reconditum* from dogs of Kerala.

Detailed clinical investigations included ECG, ultrasonography and radiography were conducted to visualize the abnormalities encountered with vital organs.

Haematobiochemical studies of dogs affected with both non sheathed and sheathed microfilariae revealed mild anaemia with severe leucocytosis, neutropenia, lymphocytosis, eosinophilia, elevated ESR and severe thrombocytopaenia, hyperproteinemia with hyperglobulinaemia and non significant reduction in AG ratio, increased serum ALT, AST, ALP , BUN and creatinine values could be observed when compared to healthy controls. Qualitative urinalysis revealed proteinuria with reduced specific gravity. Quantitative analysis of urinary markers revealed elevation of NAG, UPC, γGT and ALP in microfilaraemic dogs.

The elevated levels of serum total protein, globulin, serum enzymes like ALT and ALP and nonsignificant reduction in AG ratio suggestive of liver pathology in microfilaraemic dogs. Elevated levels of BUN, creatinine, urine protein creatinine ratio, NAG, ALP, proteinuria with low specific gravity confirmed the renal involvement in microfilaraemic dogs irrespective of the type of microfilaria involved in the disease process. This multiorgan pathology in canine microfilariosis suggested the involvement of toxic and immunological effects of these parasite in the pathogenesis of the disease.

The treatment response was evaluated by the periodic clearance of microfilariae on wet blood film examination, remission of clinical signs and the improvement in haematobiochemical alterations. Single oral dose of ivermectin @ 100 µg/kg body weight can be selected as a treatment modality for microfilariosis due to *Dirofilaria repens* and *Dipetalonema reconditum* in dogs. Levamisole hydrochloride @ 10 mg/kg body weight for seven days was the only effective treatment for microfilariosis due to *Brugia malayi* in dogs. Result of post treatment values of hepatic and renal function test revealed that many of the parameters like ALT, ALP and BUN were still in elevated level.

Two animals died during the course of treatment were subjected to post mortem examination. The gross and hispathological examination revealed lesions in heart, lungs, liver and kidneys in microfilaraemic dogs. Myofibrillar fragmentation, atlectasis, thromboemboli formation, portal hepatitis and chronic interstitial nephritis were the major lesions observed.

Based on above studies it concluded that follow-up evaluation of these parameters could be a relevant approach to find out the therapeutic effectiveness. A therapeutic plan should consists of both specific and clinically supportive treatments to improve hepatic and renal function.

Further studies are warranted to elucidate the possible role of dogs in the transmission of human filariasis and to develop a suitable therapeutic approach to treat canine microfilariosis in the increasing ewe of chronic renal diseases in dogs.

Appendix

b) Clinical Signs

(Present / Absent)

1. Lethargy :
2. Vomiting :
3. Anorexia :
4. Haemoglobinuria :
5. Oedema of any part if any :
6. Ascites :
7. Jaundice :
8. Cough :
9. Dyspnoea :
10. Syncope :
11. Muscle weakness :
12. Exercise intolerance :

5. Results of Special Examination

1. Ultrasonography Findings

i) Liver :

ii)Kidney :

2. Haemato-biochemical Findings

Sl no	Parameters	Result	
		Day 1	**Day7**
1.	Hb (gm/dl)		
2.	RBC (10^6/cu.mm)		
3.	VPRC %		
4.	ESR mm		
5.	TLC(10^3/cu.mm)		
6.	DLC		
	Neutrophils(%)		
	Lymphocytes(%)		
	Eosnophils(%)		
	Monocytes(%)		
	Basophils(%)		
7.	Platelet count		
	Serum Analysis		
	Alkaline Phosphatase (IU/L)		
	Alanine Amino Transferase(IU/L)		
	Total Protein(g/dl)		
	Albumin(g/dl)		
	A:G Ratio		
	Serum Creatinine (mg %)		
	BUN (mg%)		

ANNEXURE – I

PROFORMA

Case No. / Sl No. **Date**

1. Name And Address Of The Owner :
2. Detatils Of The Animal :
Breed :
Age :
Sex :
Colour :
If vaccinated :
If yes, Details :

3. Clinical History

Date	Diseases encountered in the past	Treatment adopted

1. General Clinical Examination :

2.Systemwise Examination :

a)Digestive System :

b)Respiratory system :

c)Cardio-vascular system :

4. Clinical Observation

a) Clinical Data

1. Respiration rate (per minute) :
2. Pulse (rate per minute) :
3. Temperature :
4. Mucous membrane : (pale/congested/icteric)
5. Lymph nodes :
6. **Wet Flim examination**

Microfilaria	**Absent**	**Present**			
		++++	+++	++	+

3. Urinalysis

Protein :

Bile pigments :

6. Diagnosis :

7. Treatment :

SIGNATURE OF THE CHAIRMAN **SIGNATURE OF THE STUDENT**