SHORT COMMUNICATION

Sensitivity of Wet Blood Smear Examination, Modified Knott's Method and Polymerase Chain Reaction for Diagnosis of *Dirofilaria immitis* Infestation in Dogs

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Abstract

The information on epidemiology and management of vector borne diseases of dogs in India is limited, despite favourable climate for parasite and vectors growth and propagation, and large population of stray dogs. The accurate identification of filarial species is clinically important because of its zoonotic importance and therapeutic recommendations. The present study was carried out to determine the sensitivity of dirofilaria species identification protocols in dogs, presented to Animal healthcare establishments in Bhubaneswar (India). The selection of dogs for screening was based on the observed clinical signs. The screening was done using conventional methods such as wet film and modified Knott's method, and polymerase chain reaction (PCR). The blood samples from 65 suspected cases were screened, out of which 38 were detected positive by conventional method, whereas 42 were positive by highly sensitive PCR method. Thus, 4 samples, negative by conventional method, were found positive for *D.immitis* in PCR assay.

Key words: *Dirofilaria immitis,* Dogs, Modified Knott's method, Polymerase chain reaction, Wet blood smear

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Introduction

Canine heartworm, *Dirofilaria immitis*, is a mosquito-borne filarial nematode that affects domestic and wild canids and felids. The parasite has public health concern because of its zoonotic significance (Noack *et al.,* 2021) This filarial nematode is transmitted by various mosquitoes belonging to genera Aedes, Culex, Anopheles, and Mansonia (Sharifdini *et al*., 2022). The filarial nematodes are characterized by tissue tropism and their dependence on blood-feeding arthropod vectors for transmission. The most commonly reported filarial species in dogs are *Dirofilaria immitis*, *Dirofilaria repens, Acanthocheilonemar econditum*, *Acanthocheilonema dracunculoides*, *Brugia malayi*, *Brugia ceylonesis* and *Brugia Pahang.* The filarial parasites reported in India include *D. immitis*, *Acanthocheilonema spp. and Brugia spp.* (Ananda and D'Souza, 2006).

The clinical signs of dirofilariasis depend on the parasitic load, stage of life cycle of the worm, and the host response to the infection, and include gradual loss of body weight, tenacious coughing, dyspnoea, lethargy, exercise intolerance, blood coughing, distended abdomen, congestive heart failure, intravascular haemolysis, and pulmonary thrombo-embolism and mortality of untreated animals (Sharifdini *et al*., 2022). Occasionally, blood flow through the lungs is obstructed due to large numbers of adults in the pulmonary arteries. The worms may migrate to the right atrium, right ventricle and vena cava, causing blockage of blood flow, known as caval syndrome, resulting in heart failure and death (Schaer, 2003). Canine heartworm disease is associated with regenerative macrocytic hypochromic anaemia and biochemical changes. The radiographic examination reveals the disease severity and cardiopulmonary changes including enlargement of right heart and damage to the pulmonary arteries in severe and chronic dirofilariasis (McCall, 2003). A survey involving postmortem examination of dogs in Odisha revealed infestation with *D. immitis* and *D. repens* in 57% and 14%, of animals, respectively (Patnaik, 1989).The present study was carried out to assess and compare different diagnostic methods of heart worm infestation in different breeds of dogs.

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Materials and Methods

Animals Selection and Screening

The dogs presented to the Veterinary Clinical Complex (VCC) of College of Veterinary Science and Animal Husbandry, Bhubaneswar and Odisha State government run Veterinary Hospital at Sahidnagar, Bhubaneswar (India), with a tropical savanna climate, for a period of eight months were included for the present study. Out of total dogs presented, 65 dogs, comprised of Labrador (n=12), Spitz (n=10), indigenous breeds/stray dogs (n=21), Doberman (n=6), GSD (n=10), and Dachshund (n=6), were suspected for dirofilariasis based on the clinical signs.

Five mL of blood sample was collected from each recruited dog from the recurrent tarsal vein. Wet blood smear examination, thick blood smear examination, modified Knott's method were employed for screening of dirofilariasis.

Molecular Diagnosis

Molecular diagnosis was performed by polymerase chain reaction to identify the type of filarial nematode,responsible for the canine heart worm disease, following the protocols of DNA extraction, primer designing, polymerase chain reaction (PCR), gel electrophoresis, low melting agarose gel electrophoresis, gel cutting, gel purification and sequence analysis. Phenol-Chloroform method was used for extraction of DNA from the blood samples. The concentration of DNA was quantified by measuring the absorbance at 260nm in a Nanodrop ND-1000 spectrophotometer (Thermo- Scientific, USA). The purity of the samples was also checked by measuring the ratio of $O.D_{260nm}$: $O.D_{230\ nm}$

Primer used in this study was a published primer (Rishniw*et al*.,2006) that spanned the internal transcribed spacer region of 5.8 s (ITS2-28s) of the ribosomal DNA, thereby differentiating six species of canine filarial nematode. The primer pair comprised DIDR- F1- AGT GCG AAT TGC AGA CGC ATT GAG and DIDR-R1 AGC GGG TAA TCA CGA CTG AGT TGA as primer sequence ordered from IDT, India. All amplification reaction consisted of initial denaturating temperature set at 94°C for 2 min followed by 32 cycles of denaturing at 94°C for 30 s and annealing at 60°C for 30 s, then extension at 72°C for 30 s. The PCR mixture (25µL) was prepared from 5µL of genomic DNA, 0.5µLdNTP, 0.5µL of forward primer and reverse primer, 2.5µL of Taq buffer and 0.25µL of Taq DNA polymerase enzyme (Genei, Merk specialities Private Limited, Bangalore) and Milli Q distilled water to make up the volume to 25µL. The mixture was spun and made ready to run PCR (96 well Thermal Cycler, Applied BiosystemVeriti). The PCR product was examined using 1% agarose gel added with 0.1% ethidium bromide.The gel electrophoresis was run at 100 volts, 400mA for 1 h after loading of the wells. The bands were viewed in alpha image software with respect to the 100 base pair marker.PCR product was further subjected to run under low melting agarose gel (SeaPlaque^{R}Agarose, LONZA) for purification of double bands. The optimal density of the PCR purified product was taken by the help of a Nanodrop ND-1000 spectrophotometer (Thermo- Scientific, USA). The purified products were sent for sequencing to Xcelris Lab Ltd, Ahmadabad, India. The sequences were confirmed by NCBI BLAST search. The alignment and analysis were interpreted using Bio Edit 7.0.0 software.

Result and Discussion

Out of 65 suspected dogs screened, 42 samples were found positive in molecular method using PCR, and 38 samples showed positive by the traditional wet film method, Modified Knott's method, and wet film test**.** The molecular diagnosis of 65 suspected samples was carried out for confirmatory analysis for dirofilariasis. All blood derived DNA samples were positive for canine beta actin, indicating adequate DNA extraction. The quantitative assay of derived DNA ranged between 1.65: 0.32 and 1.70: 0.44 at $O.D_{260nm}$: $O.D_{230nm}$ and positive for canine beta actin suggested adequate DNA extraction and less salt contamination. Out of 65 suspected samples, 38 were identified by modified Knott's method and wet film test. However, 42 samples were positive by PCR detection method including 38 samples detected positive by conventional methods. Out of 42 identified samples, 15 were single band and 26 were double band and one with single lower band (Figure 1). The upper band of double band and the single band samples were positive for *D.immitis* after sequencing the purified PCR product. The lower band was suspected for *D.repens,* which could be further verified after sequencing *D. immitis* has a cosmopolitan distribution and infects dogs, cats, and wild canids, as well as humans (Sharifdini *et al*., 2022). The present study confirmed the presence of microfilaria in the blood in 42 dogs by modified Knott's method, wet blood film examination and PCR method. Methods like wet blood smear and modified Knott's methods have been employed for detection of microfilaria in blood. It is easy to diagnose and differentiate *Dirofilaria immitis* and *Dipetalonema reconditum* through the modified Knott's method (Newton and Wright, 1971). DNA-based

Fig. 1: PCR band obtained on gel documentation

diagnostic tests for *D.immitis* infection and use of specified PCR based assay have been used as a reliable diagnostic tool for canine dirofilariasis (Rishniw*et al*., 2006).

Reliable diagnosis of filarial infections in companion animals is critical for prevention and control of disease and for monitoring the spread of these parasites that has zoonotic potential, to non-endemic areas (Genchi and Kramer, 2017). Several methods including haematological examination, serological test and molecular methods have been employed by various researchers for diagnosis of dirofilariasis in dogs (McCall, 2003; Schaer, 2003; Rishniw*et al*., 2006; Song *et al*., 2010; Pereira *et al*., 2013).Wet blood film method is a quick test requiring less blood than Modified Knott's method that helps in diagnosis by the presence of microfilaria in blood at the patient bedside and does not require much laboratory facilities for confirmation of dirofilariasis in dogs (Simsek*et al*., 2008).Modified Knott's method with the use of methylene blue stain helps in differentiation of *D. immitis* microfilaria from *Dipetalonema reconditum* by the presence of cephalic hook (Newton and Wright, 1971). Poor sensitivity of wet blood smear examination, at certain instances, can be eliminated by examination of stained blood smear under microscope (Stein and Lawton, 1973). The need to correctly identify the filarial species is becoming relevant throughout the world, because of the increased frequency of transportation of dogs between countries and continents (Zahler*et al*., 1997; Genchi and Kramer, 2017). However, morphological identification of circulating microfilaria is not always easy and is potentially misleading (Roshniw *et al.,* 2006). Several investigators have examined the molecular differentiation of microfilaria using Southern blotting to identify DNA repeats from *D. repens* (Chandrasekharan *et al*., 1994) or PCR to differentiate other filarial nematodes from D. *immitis* (Baneth *et al*., 2002; Mar *et al.,* 2022; Rishniw *et al*, 2006' Pereira *et al*., 2013).

It is concluded from the study that PCR assay was a highly sensitive method over conventional methods for diagnosis of heartworm parasite in dogs, and it can be used effectively to screen a large number of samples in one run.

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