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Research Articles

Molecular characterization of zoonotic *Dirofilaria repens* microfilariae in dogs of Kerala, India

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Abstract

Dirofilariasis in dogs is an endemic zoonotic disease, spread in the tropics, inclining to spread into the regions with moderate climate. This study presents the detection and molecular characterization of *Dirofilaria repens* microfilaria in dogs of Kerala, India. Blood samples from hundred dogs from different parts of Kerala with clinical signs suggestive of microfilariosis were screened for microfilaria by wet film examination. Out of this, 80 percent of dogs were positive for microfilariae and *Dirofilaria repens* contributes 64 percent based on parasitological studies. Molecular characterization of *Dirofilaria repens* microfilariae in dogs using pan-filarial primers revealed 94 percent homology with already published sequence of *Dirofilaria repens* in Genbank data base and was submitted to the Genbank data base at the National Centre for Biotechnology Information and assigned accession number FJ717410. Therefore, appropriate measures should be instituted to control the occurrence and distribution of filariasis in dogs as a health problem. Also the public heath aspect of this infection warrants timely prophylactic programme to be adopted in animal hosts.

Key words: *Dirofilaria repens*, Dog, Filariosis, Histochemical, PCR

Disease due to filarial nematodes is common throughout many parts of the world (East Europe, Africa, Asia) 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*. Dirofilariosis due to *Dirofilaria repens* is a helmintic zoonosis and the parasite resides in the subcutaneous connective tissues mainly of dogs. The parasite has a complex life cycle, with mosquitoes serving as intermediate hosts. Adults reside in the subcutaneous connective tissues; females produce microfilariae in the natural host and release them into the circulation. There are reports from domestic cats (Nuchprayoon *et. al*., 2006) and foxes (Gradoni *et. al*., 1980) and a large number of cases reported in humans (Pampiglione and Rivasi, 2000). *Dirofilaria* infections in humans and dogs in Kerala was described by Sabu *et al*. (2005) through microscopical examination of morphology of microfilaria in stained smears. Routine diagnosis is carried out through microscopical examination of morphology of microfilaria. However, it is difficult to differentiate various species of microfilaria because of the similarity in their morphology. However when Giemsa stain is used, it is difficult to discrete clearly between closely related species. Histochemical staining to detect acid phosphatase activity could overcome this problem (Yen and Mak, 1978) but it requires fresh sample to yield optimal results. Moreover this is time consuming and laborious and requires expertise to identify and confirm the species (Nuchprayoon, *et al*., 2006). Recent advances in molecular biology have provided scope to address these shortcomings of the traditional diagnostic methods. In this paper, morphological identification of *Dirofilaria repens* microfilariae from canine blood by routine staining techniques and its confirmation by PCR and sequence analysis is discussed.

Materials and Methods

The study was conducted in the Department of Clinical Veterinary Medicine, College of Veterinary and Animal Sciences, Mannuthy. 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. Positive cases were subjected to Giemsa and Histochemical staining techniques and molecular studies.

Giemsa and Histochemical Staining Techniques

About 3 ml of blood drawn from the cephalic vein of animals and screened positive for microfilariae

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by wet film examination was 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, air dried and were subjected to giemsa staining and histochemical staining.

For 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.

For histochemical staining, smears were 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).

Ten millilitre each of venous blood collected from sixty dogs found positive for *Dirofilaria repens* microfilariae on Giemsa and Histochemical staining were utilized for molecular studies.

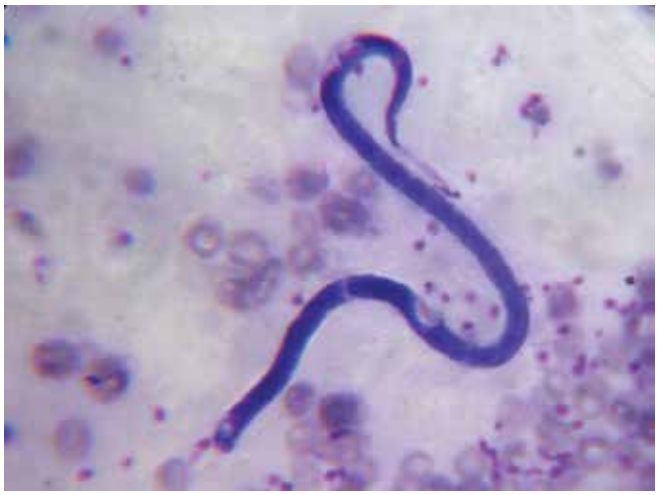

Fig. 1: Giemsa staining of *Dirofilaria repens* microfilariae (100X)

Fig. 2: Histochemical staining of *Dirofilaria repens* microfilariae (100X)

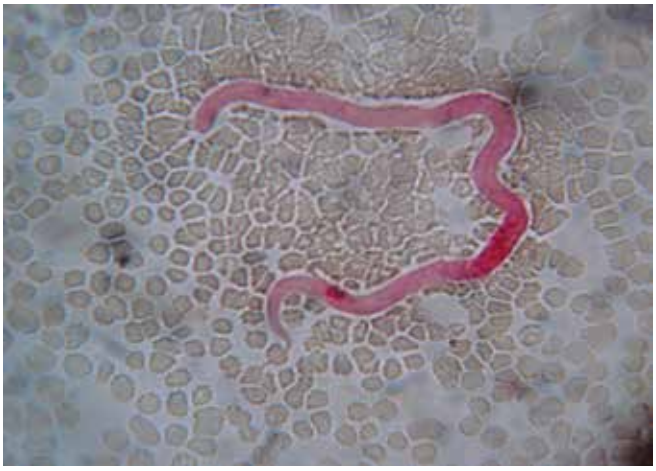

Fig. 3 : Histochemical staining of *Dirofilaria repens* microfilariae (100X)

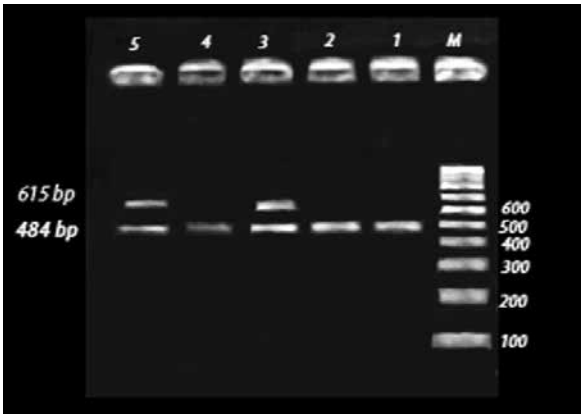

Fig.4 : PCR analysis using universal primer (DIDR-F1 and DIDR-R1) revealed two bands of 484 bp and 615 bp in case of mixed infection with *Dirofilaria repens* and *Brugia malayi* microfilariae

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 for 10 minutes. The DNA obtained were stored at –20°C for later use.

PCR amplification and Sequence analysis

A single-step multiplex PCR targeted at amplifying the internal transcribed spacer-2 region of ribosomal DNA developed by Rishniw and colleagues (2006) was utilised for molecular screening of canine filarial species in blood. Pan-filarial primers, forward: DIDR-F1 5'-AGT GCG AAT TGC AGA CGC ATT GAG-3' and reverse: DIDR-R1 5'-AGC GGG TAA TCA CGA CTG AGT TGA-3' were utilized to amplify and differentiate *D.immitis*, *D. repens*, *B. malayi*, *B. pahangi*, *A. reconditum* and *A. dracunculoides*. The primers were developed at Integrated DNA Technologies (IDT), USA.

Twenty five microliter of PCR reaction mixture was carried out by using 0.1 mM of each primer, DIDR-F1 and DIDR-R1 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 was analyzed 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).

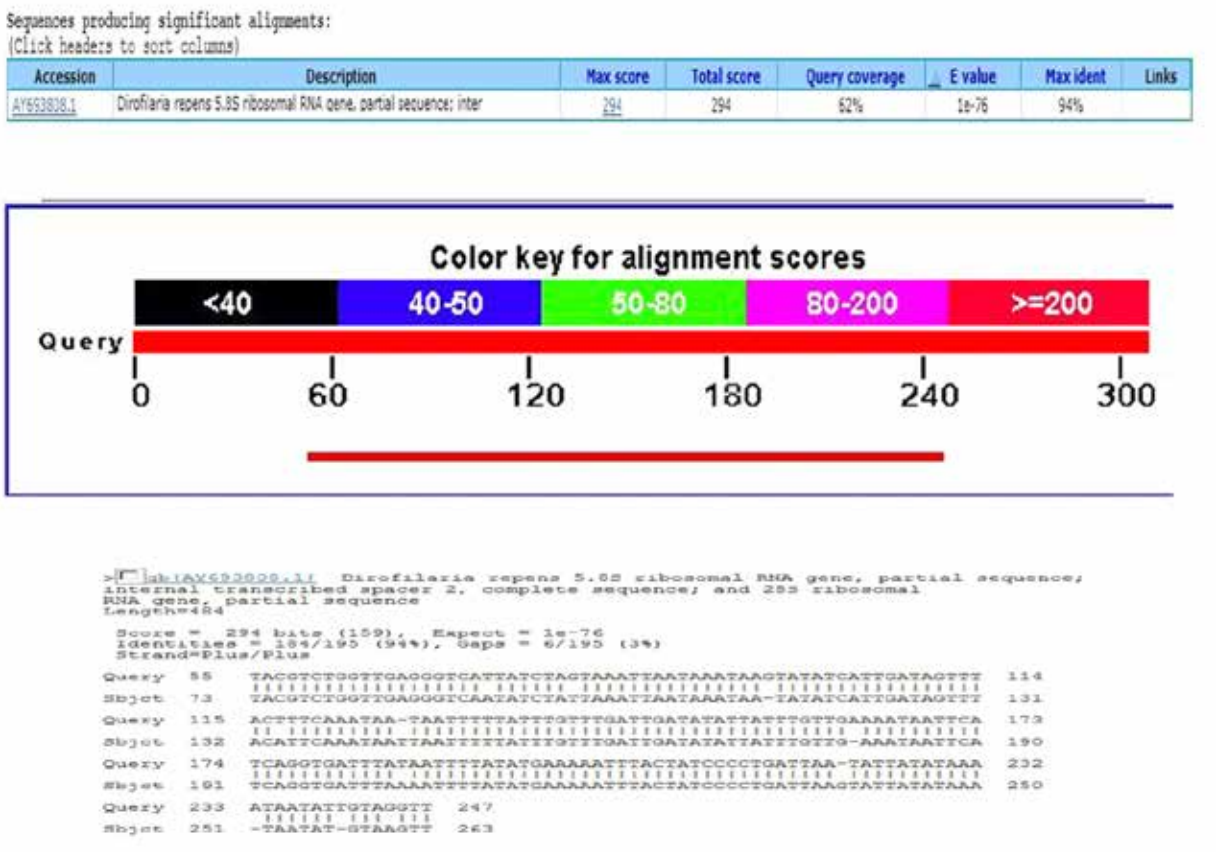

Fig. 5 : Sequence analysis of 484 bp using BLAST revealed 94% identity with already published partial sequence of 5 .8S rRNA gene and internal transcribed spacer 2 (ITS-2) region of *Dirofilaria repens* (AY693808.1)

Results and Discussion

Species identification by Giemsa and Histochemical staining

Giemsa and histochemical stained smears of microfilaraemic dogs revealed 60 out of 100 dogs were found positive for *Dirofilaria repens* microfilariae. On Giemsa stained smears, microfilariae which were sheathless with a blunt head and a long tapering tail was identified as *Dirofilaria repens* (Fig.1) (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. Histochemical staining of nonsheathed microfilaria revealed two distinct types of microfilariae. Nonsheathed microfilaria that had acid phosphatase activity at the anal pore only is in accordance with *Dirofilaria repens* (Fig.2) 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* (Fig.3) as suggested by Valcarcel *et al.* (1990) and Ananda *et al.* (2006).

Species identification by PCR and Sequence analysis

Polymerase chain reaction technology was utilized to diagnose filarial infestations in dogs and human beings by Favia *et al.* (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 (Fig.4) in case of mixed infection with nonsheathed and sheathed microfilariae which corresponds to the amplification of ribosomal DNA spacer sequences of *Dirofilaria repens* and *Brugia malayi* microfilariae as reported by Rishniw *et al.* (2006). The 484 bp amplicon obtained were sequenced and the sequences were analysed using BLAST revealed 94% identity with a query coverage of 62% with the already published partial sequence of 5 .8S rRNA gene and internal transcribed spacer 2 (ITS-2) region 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 (Fig.5)

Dirofilariasis in dogs is an endemic disease, spreads in the tropics, inclining to spread into the regions with moderate climate. With geographical spreading of infection caused by in dogs, more frequent infections in humans caused by these species of parasites should be expected. Therefore, an appropriate importance should be attached to continuous control and follow-up of the occurrence and distribution of filariasis in dogs as a health problem. This is the first comprehensive study that has utilised a combination of conventional and molecular techniques to confirm the occurance of *Dirofilaria repens* species in Kerala. The public health importance of *Dirofilaria repens* also suggests that appropriate prophylaxis be administered to dogs.

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