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Identification of *Brugia malayi* in dogs in Kerala, India

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Abstract. Identification of filarial species in dogs is clinically important because of zoonotic concerns and therapeutic implications. The present study was carried out to identify the filarial parasites causing microfilaraemia in dogs in Thrissur District, Kerala- an endemic area for human *Brugian* filariasis. Out of the 1600 dogs screened by wet blood film examination, 130 were positive for microfilariasis. Giemsa staining of blood smears revealed that 90 out of 130 dogs had unsheathed microfilariae, 24 had sheathed microfilariae and 16 had combined infection of sheathed and unsheathed microfilariae. Results of micrometry and histochemical staining of the sheathed microfilariae were in conformity with that of *Brugia malayi*. The DNA isolated from the sheathed microfilariae amplified the primers specific for the *Hha* 1 repeats of the *B. malayi*. Cloning and sequencing revealed that the amplified fragment corresponded to the 140-292 base pairs of the 320 base pair *Hha*1 repeat of *Brugia malayi*. The amplified DNA fragment also contained restriction sites for *Alu* 1 and *Rsa* 1which confirmed that the present isolate is *Brugia malayi*. The present study confirmed the presence of *B. malayi* in dogs in Kerala, India.

INTRODUCTION

Identification of filarial species in dogs is clinically important because of zoonotic concerns and therapeutic implications (Rishniw *et al.*, 2006). Dirofilariasis in dogs is usually diagnosed by identification of microfilariae in blood. Filariae such as *Dirofilaria immitis*, *Acanthocheilonema reconditum*, *Acanthocheilonema dracunculoides*, *Dirofilaria repens*, *Brugia malayi*, *Brugia pahangi*, and *Brugia ceylonensis* can cause canine microfilaremia and differentiating these microfilariae is clinically important. Infection with *A. reconditum* and *A. dracunculoides* have few clinical consequences, while *D. repens* infection has been associated with subcutaneous granulomas and pruritus in dogs. Moreover dogs infected with microfilariae of *D. repens* can act as a carrier for human infection. All canine filariae have the potential to infect humans and hence significant from a public health perspective (MegatAbd Rani *et al.*, 2010).

Dogs were not considered as reservoir hosts for *B. malayi*. Recently *B. malayi* microfilariae similar to that causing filariasis in human beings have been identified in dogs in Kerala, India (Ambily *et al.*, 2011). Contradicting the above finding, Ravindran *et al.*, 2014, reported that sheathed microfilariae from dogs in Alappuzha district, Kerala, were *B. pahangi* and *B. malayi* like microfilariae.

Detection of *Brugia malayi* in domestic dogs, in a highly endemic area for lymphatic filariasis, poses great threat to the control and eradication of human lymphatic filariasis. *Brugian* filariasis accounts for approximately 5% of lymphatic filariasis cases in India where over 40 million people are estimated to be infected with either *B. malayi* or *Wuchereria bancrofti*. The role of

dogs (and cats) as reservoirs of *Brugian* filariasis has important implications for parasite control strategies. If canine and feline reservoir hosts exist in these areas, a more inter-sectorial approach in control strategies may be required in addition to the traditional use of Mass Drug Administration (MDA) programs advocated by the World Health Organization. Therefore, accurate differentiation of canine microfilariasis and successful treatment and elimination of microfilariae from canine host is very important.

The present work was carried out to clarify the doubts on the speciation of sheathed microfilariae detected in dogs in Kerala.

MATERIALS AND METHODS

1 Selection of cases

A total of 1600 dogs above six months of age presented to the Medicine Unit of Veterinary Hospital Mannuthy and Kokkalai (Thrissur District, Kerala State, India) with various complaints, checkup and for vaccinations were screened by wet blood film examination during morning hours between 8.30 am to 12 noon. Wet film positive dogs were selected for the study.

2 Parasitological Methods

2.1 Giemsa staining

Peripheral blood smears were air dried and fixed by dipping the film briefly in a coplin jar containing absolute methyl alcohol. The film was air dried and stained with diluted Giemsa stain (1:20, vol/vol) for 20 minutes. The slide was washed in buffered water, air dried and examined under oil immersion objective of the microscope. Identification of microfilariae was done based on morphological peculiarities (Soulsby, 2005).

2.2 Micrometry

An ocular micrometer disk with 50 divisions was compared with a calibrated stage micrometer having a scale of 0.1 and 0.01 mm divisions. Low power, high power and oil immersion objectives of the microscope used for micrometry of microfilariae were calibrated using the standard method (Garcia, 2001). Measurements of microfilariae were taken using the calibrated microscope and compared with the standard measurements of the parasites (Soulsby, 2005).

2.3 Acid Phosphatase Staining

Approximately 5 ml of blood was drawn from the cephalic vein of each dog and was allowed to clot. The clot was loosened with an applicator stick and was washed with 5 ml of distilled water. The water and serum were then poured into a test tube and centrifuged for 5 minutes at 1000 x g. The supernatant fluid was discarded leaving a drop in the bottom of the tube. A drop of this fluid containing the resuspended sediment was placed on a glass slide and examined for microfilariae. Smears were prepared from the sediment were air dried, fixed in absolute acetone at 4°C for one minute and stained for the demonstration of acid phosphatase activity.

The naphthol AS-TR- phosphate method of Barka was used to demonstrate acid phosphatase activity (Chalifoux and Hunt, 1971).

3 Molecular methods

3.1 Microfilarial DNA Extraction

Microfilarial DNA was extracted using Blood Genomic DNA Miniprep Purification Spin Kit from Himedia (HiPurA, MB504) with slight modification of manufacturer's instruction. According to the manufacturer's instruction, proteinase K digestion at 56°C need be carried out only for 10 minutes. It required 16 hours of proteinase K digestion at 56°C for extraction of DNA of sheathed microfilariae.

3.2 Specific PCR for B. malayi

DNA isolated from all the samples positive for sheathed microfilariae were subjected to species specific PCR for *B. malayi*. The presence of *B. malayi* was confirmed using the primers specific for the *Hha* I repetitive sequence of *B. malayi* (Thanchomnang *et al.*, 2008). The primer sequences were

BM-F (5' TCA TTA GAC AAG GAT ATT GGT TC 3')
BM R (5' TTT AAA CTA TAA AAT GAC AAC ACA 3')

The PCR reaction contents included: 2.5 µl of 10X reaction buffer, 2.5 µl of 2mM dNTP mixture, 20 pmoles of each primer, 2 µl template DNA solution 1 unit of *Taq* polymerase and double distilled H_2O added to a total volume of 25 µl. The PCR cycling conditions used were: 95ºC, 2 minute for initial denaturation and then 31 cycles of 95ºC for one minute, 54.3ºC one minute and 72ºC for 2 minutes followed by 72ºC for 5 minutes. Ten microlitres of PCR products were analysed by using 1.5% agarose gel electrophoresis, stained by ethidium bromide and visualized under the transilluminator.

3.3 Cloning and Sequencing of PCR Products and Sequence Data Analysis

B. malayi specific, 153-bp PCR amplicon was cloned and sequenced at Bioserve, Priority Life Sciences, Hyderabad, India. The vector used was pGEM-T Easy (Promega). The sequences were analysed using BLAST and pair wise alignment with reference sequences of *B. malayi* (Accession No. M12691.1). Restriction site analysis for *Alu* I and *Rsa* I was done for the cloned sequences, reference sequences of *Brugia malayi* (No. M12691.1), *Brugia pahangi* (M12692.1), *Brugia timori* (No. AF499118) and *Hha1*sequences of human and canine microfilarial isolates (JN413104, JN601135, JN601136 and JN601137) submitted from Alappuzha, Kerala (Ravindran, *et al.*, 2014) using restriction site analysis tool 'Webcutter 2.0'.

RESULTS

Screening of dogs for microfilariae

Out of the 1600 dogs screened by wet blood film examination, 130 were positive for microfilariae. On Giemsa staining of blood smears, 90 out of 130 were having unsheathed microfilariae, 24 were having sheathed microfilariae and 16 were having unsheathed and sheathed microfilariae. Occurrence of microfilariasis in dogs was 8.1%. Occurrence of unsheathed microfilariae was 6.6% and sheathed microfilariae was 1.8%.

Parasitological Methods

In Giemsa stained smears, sheathed microfilariae had a bluish violet coloured body and a pink coloured sheath extending beyond both the anterior and posterior ends. Microfilariae were smaller in size compared to the unsheathed microfilariae and had multiple kinks over the body. The anterior end was blunt and there was a clear area devoid of nuclei called head space or cephalic space. Cephalic space was twice long compared to the width and devoid of any nuclei. Nuclear column was darkly stained and tightly packed throughout the body preventing the visualization of individual nucleus. The tail was pointed with a discontinuous row of nuclei. There were two terminal nuclei which were widely separated at the tip of the tail (Fig. 1). The nerve ring appeared as an oblique clear space devoid of nuclei, traversing the entire width of the microfilariae. Excretory pore was seen as an oval clear space opening to one side of the body. Anal pore also appeared as an oval clear space opening to one side of the body. Presence of sheath and two distinctly separated terminal nuclei confirmed it as microfilariae of genus *Brugia*. The size of the microfilariae and proportion of cephalic space suggested that the microfilariae were similar to human filarial parasite *B. malayi*.

The microfilarial length varied from about 190 to 220 micrometer (µm) with a mean of 215.0 µm and width of 4.35 to 6.52 µm with a mean of 5.0 µm. The cephalic space ranged from 8.6 to 10 micrometer with a mean of 9.50 µm. The nerve ring appeared as an oblique clear space devoid of nuclei, traversing the entire width of the microfilariae at around 45.35 to 48.50 micrometer with a mean of 46.0 µm from the anterior end. Excretory pore was seen as an oval clear space opening to one side of the body at about 61.88 to 78.50 micrometer with a mean of 72.5 µm from anterior end. Anal pore also appeared as an oval clear space opening to

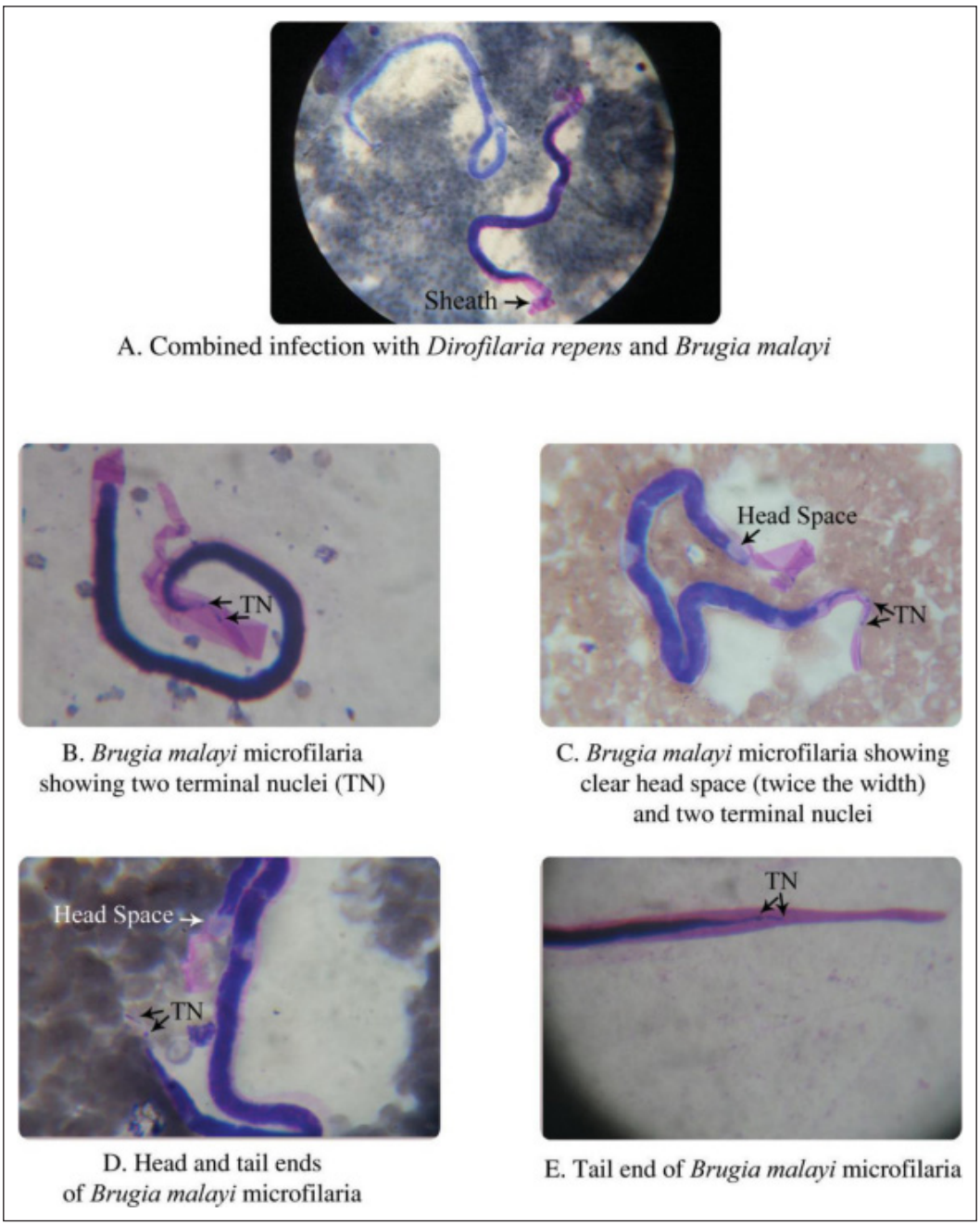
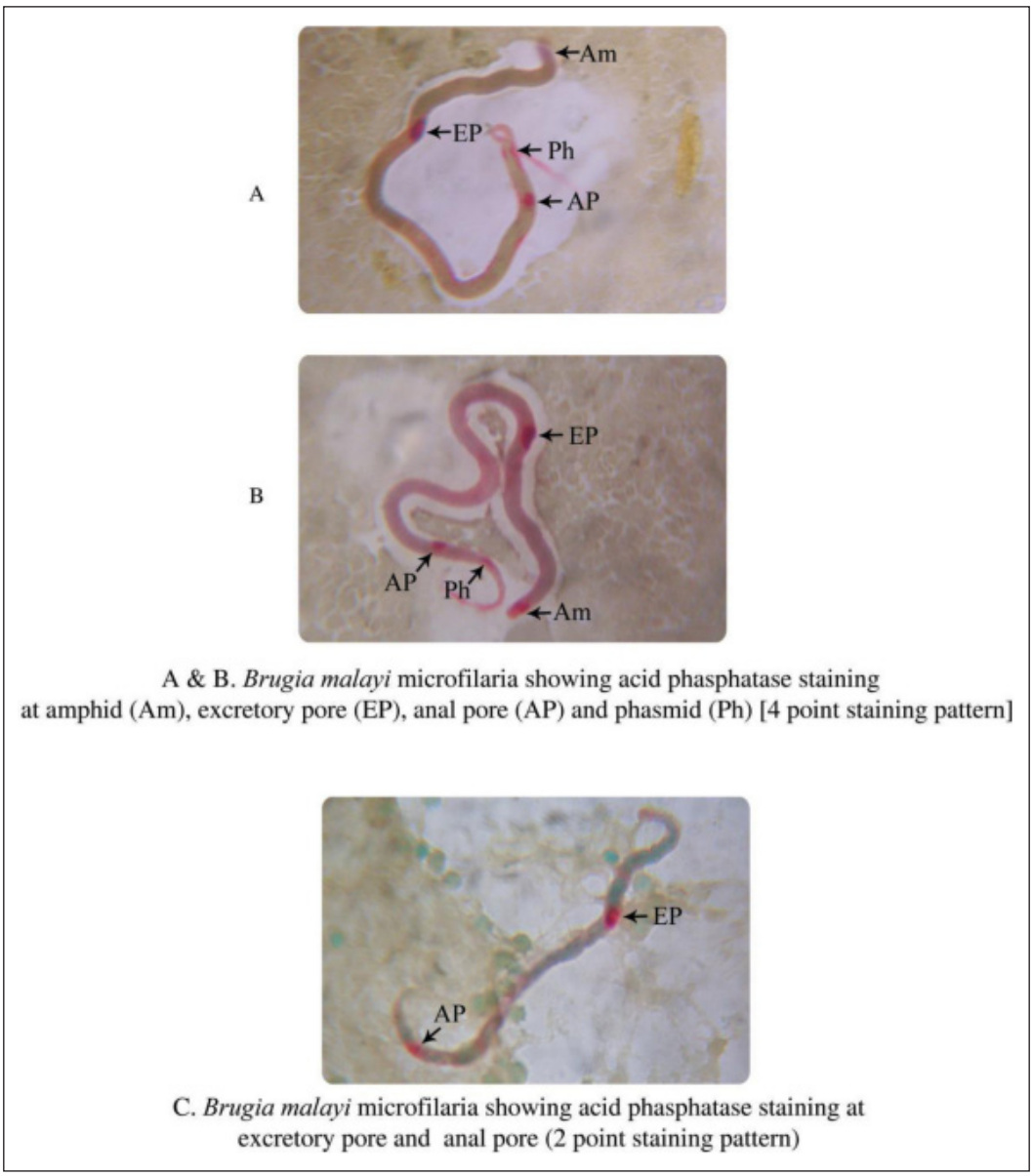


Figure 1. Giemsa staining of microfilariae.

one side of the body at about 171.88 to 184.50 micrometer with a mean of 180.00 µm from the anterior end.

Histochemical staining of sheathed microfilariae revealed two staining patterns. In one type, four points in the microfilariae have taken bright red colour, indicating the acid phosphatase activity in these four areas (Fig. 2). The four regions were amphids, excretory pore, anal pore and phasmid. Acid phosphatase activity at excretory pore and anal pore appeared as oval red spots opening to one side of the body of microfilariae. Acid phosphatase activity at amphid and phasmid



A & B. *Brugia malayi* microfilaria showing acid phasphatase staining at amphid (Am), excretory pore (EP), anal pore (AP) and phasmid (Ph) [4 point staining pattern]

C. *Brugia malayi* microfilaria showing acid phasphatase staining at excretory pore and anal pore (2 point staining pattern)

Figure 2. Acid phasphatase staining of sheathed microfilariae.

appeared as two small red lines resembling the sign. Second type showed staining at two points, *viz.*, excretory pore and anal pore as red spots. Among the 24 cases with sheathed microfilariae alone, 21 (87.5%) revealed four point staining pattern and 3 (12.5%) revealed two point staining pattern. The histochemical staining pattern suggested that the microfilariae were similar to human filarial parasite *B. malayi*.

Molecular methods

Primers specific for *B. malayi* amplified a fragment having a size between 100 and 200 bp from all the 30 samples containing sheathed microfilariae (Fig. 3).

Sequencing the cloned fragment revealed the presence of 153 bp and the sequences were analysed using NCBI BLAST. Analysis showed 97% identity with *B. malayi* isolate 1 Alappuzha (Accession No. JN413104.1)

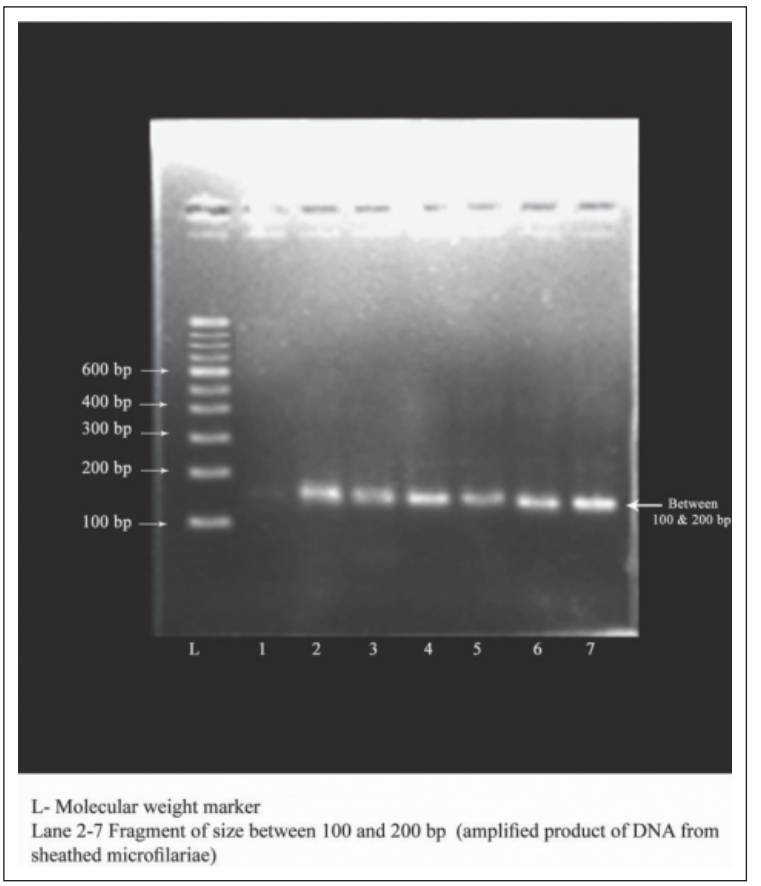


Figure 3. Gel electrophoresis of filarial PCR products using *B. malayi* specific primers.

Hha I repeat region (which was isolated from human samples in Alappuzaha district of Kerala) and 96% identity with *B. malayi* ex. canine Kadakkarappally 2 (Accession No. JN601137.1) *Hha* I repeat region (which was isolated from canine samples in Kadakkarapally region of Alappuzha district). With 100% query coverage, sequences of the present isolate showed 93% identity with the reference sequences of *B. malayi* (Accession No. M12691.1).

Pair wise sequence alignment of the 153 base pair sequences with reference sequence of *B. malayi* (Accession No. M12691.1), presented in Fig. 4, revealed 93% sequence identity. The sequences revealed a deletion of 7 bases at position 65 to 71 and a transversion mutation at positions 101 and 102 base pairs.

Restriction Analysis

Restriction endonuclease analysis is presented in Fig. 5. There were three restriction endonuclease (RE) sites, two for *Alu* I and one for *Rsa* I in the 322 bp *Hha* I repeat region of *B. malayi* reference sequence (Accession No. M12691.1). The 153 bp *Hha* I repeat sequence of the present isolate (Mannuthy) also showed one site each for these two enzymes, which is about 38 bp apart, similar to that in the reference sequence. Absence of the second *Alu* I site is due to a transversion mutation (Fig. 4).

Compared to the present isolate, *Hha* I repeat region of *B. malayi* isolate from human beings of Alappuzha (Accession No. JN 413104) also contained one site each for *Alu* I and *Rsa* I but at a different region of the sequence (Fig. 5). Restriction sites of the two

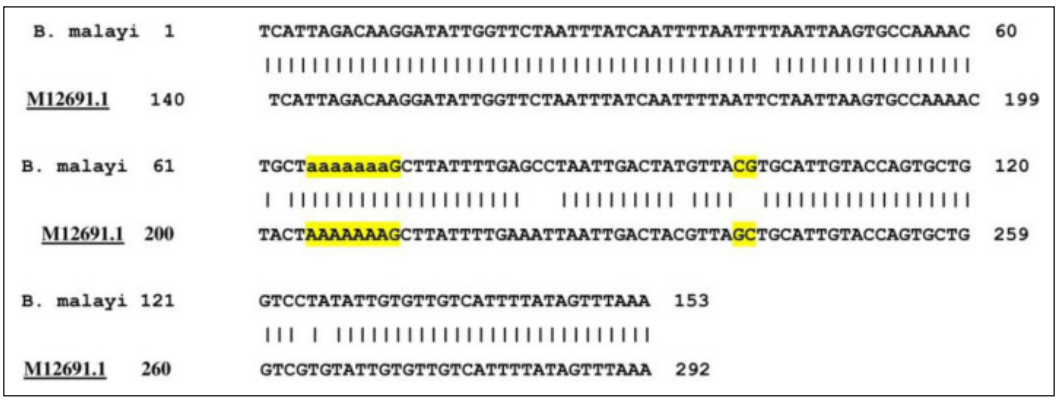


Figure 4. Pair wise sequence alignment of *B. malayi* Hha 1 repeat region with reference sequences.

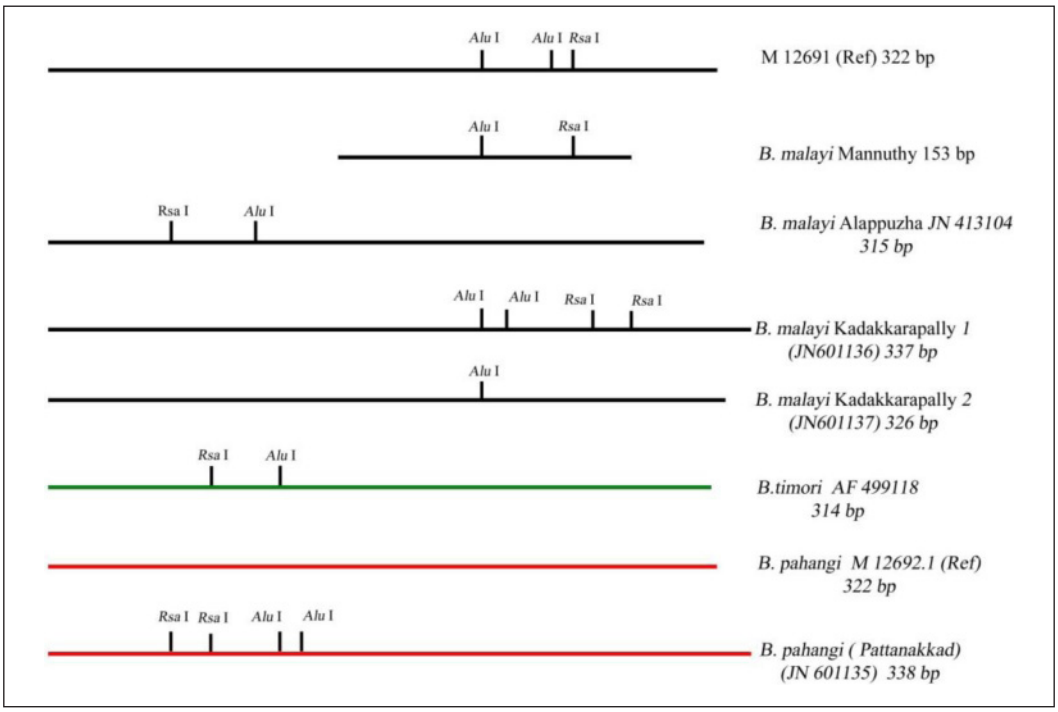


Figure 5. Restriction map of *Brugia* isolates (*Alu* I and *Rsa* I).

enzymes in *B. timori* also showed a similar location to that of the Alappuzha isolate. Two RE sites each for *Alu* I and *Rsa* I were observed in *B. malayi* isolate from canine (Kadakkarappally 1) whereas the other *B. malayi* isolate from canine (Kadakkarapally 2) contained only one site for *Alu* I alone in the region similar to that of the present isolate (*B. malayi* Mannuthy).

Analysing the *B. pahangi Hha* I repeat sequence of reference isolate (Accession No. M12692.1), revealed a total absence of RE sites for both *Alu* I or *Rsa* I. This difference in the cleavage sites of REs, *Alu* I and *Rsa* I in the repetitive DNA, allow to distinguish *B. malayi* and *B. pahangi*. But in the repeat DNA of *Brugia* isolated from Pattanakkad (Alappuzha) [Accession No. JN601135]

named as *B. pahangi*, has two RE sites each, for *Alu* I and *Rsa* I, in the region somewhat similar to that of *B. malayi* (Alappuzha) and *B. timori*.

The difference in the cleavage sites of REs, *Alu* I and *Rsa* I in the repetitive *Hha* 1, allowed to distinguish *B. malayi* and *B. pahangi* and confirmed that the canine sheathed microfilariae detected in the present study were *Brugia malayi* itself.

Parasitological studies and molecular studies confirmed that the sheathed microfilariae detected in dogs are that of *B. malayi*. The presence of microfilariae, in peripheral blood in morning hours between 8.30 am to 12 noon indicates that they are subperiodic strain of *B. malayi*.

DISCUSSION

In the present study, the occurrence of microfilariasis in dogs in Thrissur district, Kerala was found to be 8.1%. The reported occurrence of microfilariasis in dogs in Thrissur was 7.5% (Radhika, 1997) and 7% (Sabu *et al*., 2005). The present study also agrees with the above findings. Therefore screening for microfilaria must be made mandatory for all canine cases.

Presence of sheath and two distinctly separated terminal nuclei in Giemsa stained smears of microfilariae indicated that they belong to the genus *Brugia*. The size of the microfilariae and proportion of cephalic space led to the conclusion that the microfilariae were similar to sub periodic form of the human filarial parasite *B. malayi* rather than the canine filarial worm *Brugia pahangi* (Schacher, 1962; Chansiri *et al.*, 2002; Soulsby, 2005 and Ambily *et al*., 2011). Histochemical staining of sheathed microfilariae revealed two staining patterns. In one type, four points in the microfilariae took bright red colour, indicating acid phosphatase activity in these four areas. The four regions were amphids, excretory pore, anal pore and phasmid. The second type showed staining at two points, *viz*., excretory pore and anal pore as red spots. Both these types of staining patterns were reported for *B. malayi* (Ufomadu, 1990; Kanjanopas *et al*., 2001; Kobosa *et al*., 2004 and Ambily, 2009). Histochemical staining pattern of *B. pahangi* is different from that of *B. malayi*. *Brugia pahangi* microfilariae show heavy and diffused acid phosphatase activity along the entire body (Yen and Mak, 1978, Kobosa *et al*., 2004 and Nuchprayoon *et al*., 2006).

Amplification with *B. mlalayi* specific primers (Thanchomnang *et al*., 2008) produced a fragment between 100 to 200 bp. Sequencing revealed it as a 153 bp fragment and its analysis helped to distinguish *B. malayi* from *B. pahangi*. McReynolds *et al.* (1986) analysed the 320 bp repeat DNA sequences from *B. malayi* and *B. pahangi* and showed that the differences between the *Hha* I repeated sequences of *B. malayi* and *B. pahangi* could be used to distinguish the two species by restriction site polymorphisms and by differences in specific regions of the DNA sequence. The differences in DNA sequences between *B. malayi* and *B. pahangi* were not random, but are clustered in two regions, between 140-154 bp and 231-275 bp. Comparison of these two regions between the species showed only 60–65% homology. Although the *Hha* I repeats of *B. malayi* and *B. pahangi* have an average homology of 89%, half the differences are clustered in a region of 66 nucleotides that has a homology of only 72%. Similar observations were also reported by Williams *et al.* (1988). The 153 bp fragment obtained in this study encompasses the two clustered region of *Hha* I repeat DNA, harboring species specific differences between *B. malayi* and *B. pahangi*.

In the present study, analysis of the 153 bp fragment revealed 97% and 96% similarity with *Hha* I repeat region of *B. malayi* isolate from human blood sample and canine blood sample respectively of Alappuzha region, Kerala. With human *B. malayi* reference sequences (Accession No. M12691.1) and *B. timori*, 93% identity was observed. Pair wise sequence alignment with reference to *B. malayi Hha* I repeat region of 322 bp revealed that the 153 bp correspond to a region of 140-292 bp. McReynolds *et al.* (1986) showed that the differences in DNA

sequences between *B. malayi* and *B. pahangi* are clustered in two regions, between 140-154 bp and 231-275 bp of a 322 bp *Hha* I repeat. Thus, the amplified product in the present study encompassed the most divergent regions of *Hha* I repeats of the two species. The primers used here is species specific for *B. malayi* and hence, the amplification was specific to the *Hha* I repeats of *B. malayi*. Sequence alignment also showed a deletion of 7 bases at positions 65 to 71, which might have contributed to the adaptation of the parasite to the new host (dogs).

Further, restriction endonuclease analysis of the 153 bp *Hha* I repeat region obtained in the present study revealed RE sites for *Alu* I and *Rsa* I separated by a distance of 38 bp in the positions exactly same as that of the reference *B. malayi* sequence. One *Alu* I site in the present isolate is deleted due to a transverse mutation. This might also play a role in the adaptation of the parasite in the new host. McReynolds *et al.* (1986) reported that RE cleavage sites in the repetitive DNA allow to distinguish *B. malayi* and *B. pahangi*. They showed that *B. malayi* repetitive DNA sequence is restricted by *Alu* I and *Rsa* I but *B. pahangi* sequence is not. Hence, it is suggested that the 153 bp fragment obtained in the present study is a part of *Hha* I repeat DNA of *B. malayi* microfilariae and not that of *B. pahangi*. Comparison of RE sites of the two enzymes in the *Hha* I repeat DNA of the present isolate and published *B. malayi* and *B. timori* isolates revealed the presence of RE sites at similar positions in the *B. malayi* isolate from canine, Kadakkarapally 1. Whereas, the RE site of *B. malayi* from human beings (Alappuzha) were at a different region of the *Hha* I repeat DNA, similar to that of *B. timori*, there was a total absence of RE sites for these enzymes in *B. pahangi* (Reference Sequence). However, the presence of RE sites for these enzymes (two sites for each enzyme) in this region of *Brugia* isolate from Pattanakkad (Alapuzha) in a region similar to that of *B. malayi* (Alappuzha) and *B. timori* (Reference Sequence) and naming it as *B. pahangi* (Ravindran *et al.*, 2014) is to be re-examined. Xie *et al.* (1994) reported that the *Hha* I repeat DNA of *B. malayi* and *B. timori* were to be virtually identical. These two species are morphologically and molecularly very closely related among the *Brugia* species.

All the above findings such as Giemsa staining morphology, acid phosphatase staining pattern,species specific amplification of a 153 bp fragment and RE sites for *Alu* I and *Rsa* I and their similar location to that of reference *B. malayi* sequences clearly identified the present isolate as *B. malayi*. Even though, the natural host for *B. malayi* is human beings, sequence variations such as deletion of seven base pairs and a transversion mutation, which deleted an *Alu* I site might have influenced it's adaptation in dogs.

B. malayi microfilariae are of three physiological forms, nocturnally periodic, nocturnally sub periodic or even diurnally sub periodic. In nocturnally periodic form microfilariae appear in peripheral blood in peak levels at midnight, in nocturnally sub periodic, microfilariae present in peripheral blood throughout the day but in high concentration from the afternoon onwards. In diurnally sub periodic, microfilariae present in peripheral blood throughout the day but in high concentration in the morning hours (Denham and McGreevy, 1977). From India, only nocturnally periodic form of *B. malayi* has been reported in human beings (Sabesan *et al.*, 2010). In *Mastomys natalensis*, *B. malayi* exhibited diurnal subperiodicity indicating that in a different host one form of microfilariae can change its periodicity (Reddy *et al.*, 1984). Sub periodic form of *B. malayi* were detected in animals such as leaf monkeys (Nutman, 2001). In the present study also, *B. malayi* microfilariae identified in dogs were of subperiodic form. The study confirmed the presence of *B. malayi* microfilariae in dogs in Kerala, India and documented detailed description of morphological and molecular features of the microfilariae.

REFERENCES

Ambily, V.R. (2009). *Clinico-therapeutic studies on canine microfilariasis*. M.V.Sc. thesis, Kerala Agricultural University, Thrissur, 137p.

Ambily, V.R., Pillai, U.N., Arun, S., Pramod, S. & Jayakumar, K.M. (2011). Detection of human filarial parasite *B. malayi* in dogs by histochemical staining and molecular techniques. *Veterinary Parasitology* **181**: 210-214.

Chalifoux, L. & Hunt, R.D. (1971). Histochemical differentiation of *D. immitis* and *D. reconditum*. *Journal of American Veterinary Medical Association* **158**: 601-605.

Chansiri, K., Tejankura, T., Kwaosak, P., Sarataphan, N., Phantana, S. & Sukhumsirichart, W. (2002). PCR based method for the identification of zoonotic *B. malayi* microfilariae in domestic cats. *Molecular and Cellular Probes* **16**: 129-135.

Denham, D.A. & McGreevy, P.A. (1977). Brugian filariais: Epidemiological and experimental studies. *Advances in Parasitology. Volume 15* (Dawes, B.) Academic Press Inc. London. 244-296.

Garcia, L.S. (2001). *Diagnostic Medical Parasitology*. Fourth edition. ASM Press, Washington D.C. 723p.

Kanjanopas, K., Choochote, W., Jitpakdi, A., Suvannadabba, S., Loymak, S., Chungpivat, S. & Nithiuthai, S. (2001). *B. malayi* in a naturally infected cat from Narathimat Province, Southern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **32**: 585-587.

Kobosa, T., Thammapalo, S., Suvarnadabba, S., Armesombun, A., Loymak, S., Leemingsawat, S. & Choochote, W. (2004). Identification of *B. malayi*- like microfilariae in naturally infected cats from Narathiwat Province, Southern Thailand. *Journal of Tropical Medicine and Parasitology* **27**: 21-25.

MegatAbd Rani, P.A., Irwin, J.P., Gatne, M., Coleman, G.T., Mcinnes, M.L. & Traub, R.J. (2010). A survey of canine filarial disease of veterinary and public health significance in India. *Parasites and Vectors* **3**: 30.

McReynolds, L.A., DeSimone, S.M. & Williams, S.A. (1986). Cloning and comparison of repeated DNA sequences from the human filarial parasite *B. malayi* and the animal parasite *B. pahangi*. *Proc. Natl. Acad. Sci.* **83**: 797-801.

Nuchprayoon, S., Junpee, A., Nithiuthai, S., Chungpivat, S., Suvannadabba, S. & Poovorawan, Y. (2006). Detection of filarial parasites in domestic cats by PCR-RFLP of ITS1. *Veterinary Parasitol*ogy **140**: 366-372.

Nutman, T.B. (2001). Blood borne filarial infections: *Wuchereria bancrofti*, *B. malayi*, *Brugia timori*, *Loa loa*, *Mansonella perstans* and *Mansonella ozzardi*. Principles and practices of clinical parasitology. (Gillespie, S. and Pearson, R.D.) John Wiley & Sons Ltd, London. 433-456.

Radhika, R. (1997). Prevalence, clinical pathology and treatment of microfilariasis in dogs in Thrissur.M.V.Sc thesis. Kerala Agricultural University, Thrissur. 122 p.

Ravindran, R., Varghese, S., Nair, S.N., Balan, V.M., Lakshmanan, B., Ashruf, R.M., Kumar, S.S., Gopalan, A.K., Nair, A.S., Malayil, A., Chandrasekhar, L., Juliet, S., Devada, K., Ramachandran, R., Regu, K. & Showkath Ali, M.K. (2014). Canine filarial infections in a human *Brugia malayi* endemic area of India. *BioMed Research International*. 1-9.

Reddy, A.B., Chandrashekar, R., Rao, U.R. & Subrahmanyam, D. (1984). Microfilarial periodicity in *Mastomys natalensis*. *Journal of Helminthol*ogy **58**: 117-121.

Rishniw, M., Barr, S.C., Simpson, K.W., Frongillo, M.F., Franz, M. & Alpizar, J.L.D. (2006). Discrimination between six species of canine microfilariae by a single polymerase chain reaction. *Veterinary Parasitology* **135**: 303-314.

Sabesan, S., Vanamail, P., Raju, K.H.K. & Jambulingam, P. (2010). Lymphatic filariasis in India: Epidemiology and control measures. *Journal of Postgraduate Medicine* **56**: 232-238.

Sabu, L., Devada, K. & Subramanian, H. (2005). Dirofilariasis in dogs and humans in Kerala. *Indian Journal of Medical Research* **121**: 691-693.

Schacher, J.F. (1962). Morphology of the microfilariae of *B. pahangi* and of the larval stages in the mosquito. *The Journal of Parasitol*ogy **48**: 679-692.

Soulsby, E.J.L. (2005). *Helminths, Arthropods and Protozoa of Domesticated Animals.* Seventh edition. The English Language Book Society and Bailliere Tindall, London. 809p.

Thanchomnang, T., Intapan, P.M., Lulitanond, V., Choochote, W., Manjai, A., Prasongdee, T.K. & Maleewong, W. (2008). Rapid detection of *B. malayi* in mosquito vectors using a Real-time Fluorescence Resonance Energy Transfer PCR and melting curve analysis. *American Journal of Tropical Medicine and Hygiene* **78(3)**: 509-513.

Ufomadu, G.O. (1990). Localisation of acid phosphatase in *Wuchereria bancrofti* microfilaria from savanna, Nigeria. *Medical Science Research* **18**: 501.

Williams, S.A., DeSimone, S.M. & McReynolds, L.A. (1988). Species specific oligonucleotide probes for the identification of human filarial parasites. *Molecular and Biochemical Parasitol*ogy **28**: 163-169.

Xie, H., Bain, O. & Williams, S.A. (1994). Molecular phylogenetic studies on *Brugia* filariae using *Hha* I repeat sequences. *Parasite* **1**: 255-260.

Yen, P.K. & Mak, J.W. (1978). Histochemical differentiation of *Brugia*, *Wuchereria*, *Dirofilaria* and *Breinlia* microfilariae. *Annals of Tropical Medicine and Parasitology* **72**: 157-162.