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ORIGINAL ARTICLE

Canine *Brugia malayi* microfilarial excretory/secretory protein-based antibody assay for the diagnosis of brugian filariasis in dogs

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Abstract Brugian filariasis is reported in dogs in Kerala, India. Antibody detection kits are not available worldwide, for detection of Brugian filariasis in dogs. A study was carried out to develop Indirect plate ELISA using excretory secretory antigen isolated from canine brugian microfilariae and compare the sensitivity and specificity with that of blood smear examination. Identification of microfilariae was done by acid phosphatase staining using Naphthol AS-TR method and Polymerase Chain Reaction for *Hha* 1 repeat sequence. The microfilariae were identified as *Brugia malayi*. Isolation of brugian microfilariae from canine blood was done by gradient centrifugation method. The isolated microfilariae were maintained in RPMI-1640 media. The pooled media was then concentrated to obtain excretory secretory protein (ESP). This ESP was used to develop Indirect ELISA. The sensitivity and specificity of the plate ELISA developed was 84 and 100 per cent respectively when compared with blood smear examination. This is the first report of successful isolation of ESP from *Brugia malayi* microfilariae from dogs and standardization of plate ELISA using the antigen.

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Introduction

Lymphatic filariasis is common vector borne disease of humans, endemic in 250 districts in India. It is an infection caused by nematodes of the superfamily Filarioidea. Human brugian filariasis is endemic in six states in India (Srivastava et al. 2014). *Brugia malayi* microfilariae are detected in dogs also (Ambily et al. 2011; Chirayath et al. 2015, 2017). The very presence of the parasite in the same geographic location as those found in humans, and it's transmission by the same vector species indicates a possible zoonotic potential. The current approach for successful elimination of the disease may have to be modified to include the new factors as well. Early identification of the disease is vital to ensure a break in the transmission. Diagnosis is essential for the mapping of disease prevalence and for understanding the correlation with the human disease. Conventionally, the detection of filarial infections relied exclusively on the detection and identification of microfilariae. Thick blood smear examination is the routine method used for diagnosis and prevalence studies in lymphatic filariasis. However, differentiation of the species by parasitological methods requires expertise and time. This is not suitable for screening a larger population.

Several assays targeting the filarial specific IgG and IgG4 antibodies have been made using somatic antigen, excretory secretory proteins and recombinant proteins from various species of adult and pre-larval stages of *Dirofilaria* in dogs. However no such antibody detection tests are available for brugian filariasis detection in canines.

Therefore the current study was carried out to develop Indirect plate ELISA using antigen isolated from canine brugian microfilariae and compare the sensitivity and specificity with that of blood smear examination.

Materials and methods

Sample collection

A total of 3000 dogs above the age of 4 months presented to the University Veterinary Hospital Kokkalai and Teaching Veterinary Clinical Complex, Mannuthy for various complaints, general check-up or vaccination were screened by wet blood film examination for moving microfilariae during 8:00 am to 4:30 pm. Thick blood smears were prepared from wet film positive animals and subjected for Giemsa staining. The slides which were positive for sheathed microfilariae were then subjected to acid phosphatase staining (Chalifoux and Hunt 1971). Dogs which were positive for Brugian filariasis according to the staining characteristics were selected. Seventy dogs were positive for Brugian filariasis. Serum from 50 dogs positive for Brugian filariasis were collected and stored at − 20 °C for ELISA test. Serum was collected from 50 amicrofilaraemic dogs below the age of 4 months as negative control for ELISA test. Twenty milliliter of blood was collected in EDTA from three dogs each, heavily positive for Brugian microfilariae were used for excretory secretary protein (ESP) preparation.

Excretory secretary protein preparation (ESP)

Genus identification of microfilariae used for antigen preparation was done by Polymerase chain reaction (PCR) using specific primers.

Polymerase chain reaction

Genomic DNA was extracted using DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The presence of *Brugia* spp. was confirmed using the primers specific for the *Hha* 1 repeat sequences (Xie et al. 1994). PCR products obtained were sequenced at SciGenome, Kakkanad, India and the sequences obtained were then analyzed using Basic Local Alignment Search Tool (BLAST) and submitted to GenBank.

Nucleotide sequence data reported in this paper are available in the GenBank™ under the accession number: MG063782.

Isolation of microfilariae

Isolation of microfilariae from blood was done using gradient centrifugation method. Whole blood was collected in EDTA from dogs positive for brugian microfilariae. Twelve milliliters of EDTA blood was diluted with equal volume of PBS. Twelve milliliters of diluted blood was carefully layered over 3 ml of HiSep™ 1077 (Himedia, Mumbai, India) in a 15 ml centrifuge tube and centrifuged at room temperature for 30 min at 400 g in a swinging-bucket rotor without brakes. Four layers were formed; the top layer was clear and contained PBS with plasma, which was discarded. The next layer containing mononuclear cells was pipetted out. The third layer constituted of HiSep with microfilariae and some RBC contamination. The last layer was sedimented erythrocytes, in which some microfilariae were found trapped. The HiSep layer was collected in another centrifuge tube and diluted with PBS. This was centrifuged at 400 g for 5 min at room temperature and the microfilarial pellet was collected after discarding the supernatant. The wash was repeated twice more with PBS followed by a final wash in RPMI-1640 (Himedia, Mumbai, India)-AT171-1L), to get rid of the cellular contaminants.

Extraction of excretory-secretory protein

The microfilarial pellet was re-suspended in RPMI-1640 supplemented with 1% Antibiotic–Antimycotic Solution (Gibco, Thermo Fisher Scientific, Bangalore, India) such that each ml of the media had 100 U of penicillin, 100 µg of streptomycin and 0.25 µg of amphotericin B. The culture was incubated at 37 °C in a stoppered conical flask. The spent media was collected every 12 h and fresh media was provided for 48 h. The spent media was filtered using Millipore 0.22 µm to obtain excretory secretory protein (ESP) and stored at – 50 °C. The pooled samples were centrifuged in Amicon® Ultra-15 centrifugal filter device with 3 kDa cut-off membrane (Sigma-Aldrich, St. Louis, USA) at 4000 g for 45 min at 12 °C. The supernatant constituted the concentrated protein and was stored at – 50 °C until further use. Protein concentration was estimated using the Nanodrop 2000/2000c (Thermo Fisher Scientific, Bangalore, India) at 595 nm wavelength by Bradford's assay.

Indirect plate ELISA

Standardisation

Checkered board titration method was used to find the optimum concentration of the antigen, test serum samples and anti-canine IgG-HRP conjugate (Thermo Fisher

Scientific, Bangalore, India). Serum from one dog, out of the three which were heavily positive for Brugian microfilariae, used for antigen preparation, was taken as the positive control. Serum from a 3 month old dog which was negative on blood smear examination and PCR was used as negative control. The ELISA was performed using antigen concentration of 600 ng, 300 ng, 150 ng, 75 ng, 37.5 ng and 17.25 ng. Positive and negative serum samples were used at 1:100, 1:200, 1:300 and 1:400 dilutions. The anti-canine IgG HRP conjugate was used at 1:10,000, 1:15,000 and 1:20,000 concentrations. The optimum concentration was found to be 300 ng, 1:300 and 1:20,000 for the antigen, sera and conjugate respectively.

Determination of cut-off values

The cut-off values for interpretation were determined as per the report of Bomfim et al. (2005). The mean OD with 50 negative sera collected from canines below the age of 4 months was recorded. The cut-off value was equal to the three times the standard deviation in addition to the mean OD obtained.

Test proper

The test proper was performed using samples which were collected from brugian microfilaria positive animals (n = 50) and negative samples (n = 50). ELISA plate with 96 wells was sensitized with 100 µl antigen ESP overnight at 4 °C (300 ng ESP per well in carbonate-bicarbonate buffer, pH 9.6). The antigen solution was aspirated and the plates were washed 3 times with PBST. Wash was repeated as described between every two steps. The wells were blocked using 100 µl of the blocking solution per well for 1 h at 37 °C. 100 µl of serum of 1:300 dilution was added to each well and incubated for 1 h at 37 °C. At the ideal dilution, 100 µl of peroxidase labeled anti-canine IgG was added to each well and incubated at 37 °C for 1 h. 100 µl of 1X TMB substrate solution was added to the conjugate and incubated in the dark for 10 min at room temperature. 100 µl of 1 M H_2SO_4 was added to each well to stop the reaction. The OD was calculated at 450 nm and recorded.

Analysis of data

Statistical analysis of the difference between blood film examination, indirect ELISA and Dot ELISA was done by Chi square test.

Results

Identification of microfilariae

A total of 3000 dogs above the age of 6 months presented to the University Veterinary Hospital, Mannuthy and Kokkalai were screened for microfilariae by wet blood film examination over a period of one year. Blood smears from positive cases were subjected to Giemsa staining. Seventy cases were positive for sheathed microfilariae. Sheathed microfilariae revealed a pink sheath, the cranial end had head space and the caudal end showed two clear distinct tail nuclei (Fig. 1). Histochemical staining of sheathed microfilariae revealed a four point staining pattern with acid phosphatase activity at the phasmid, anal pore, excretory pore and amphid (Fig. 2). These regions were bright red points which were distinctly visible even under

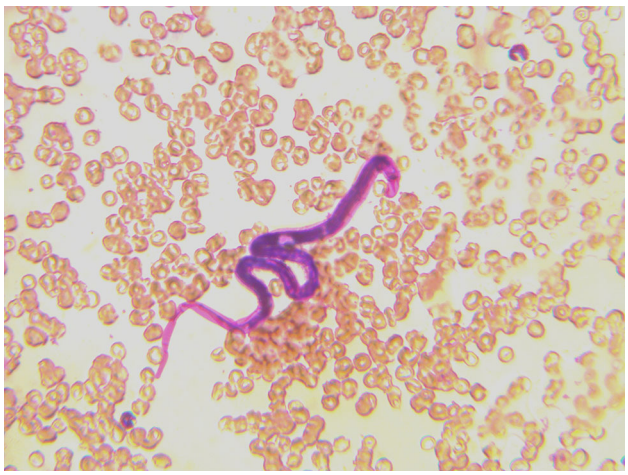

Fig. 1 Giemsa staining of *Brugia malayi* microfila showing pink sheath

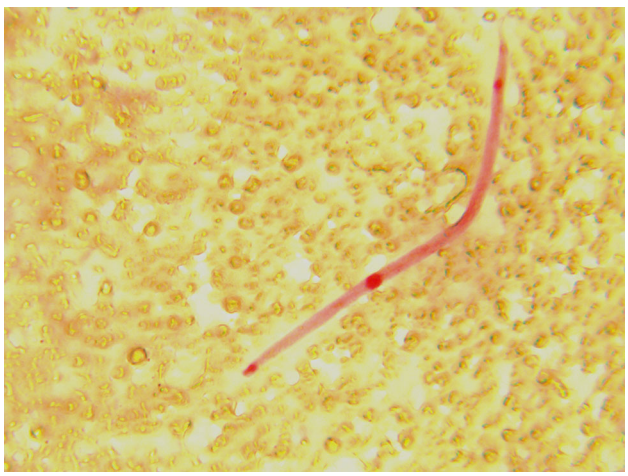

Fig. 2 Acid phosphatase staining of *Brugia malayi* microfilaria showing four point staining pattern

low power. Genus identification of microfilariae used for antigen preparation was done by PCR using specific primers for *Hha* 1 repeats. Amplification of the template with *Hha*1 primers gave an amplified product of 322 bp. The product obtained was sequenced and analyzed using Basic Local Alignment Search Tool (BLAST). The product revealed 99 per cent homology with *B. malayi* isolate (Accession No. JN601137.1) with a query coverage of 100 per cent. Nucleotide sequence data reported in this paper are available in the GenBank™ under the accession number: *MG063782* .

Preparation of excretory secretory protein (ESP)

For antigen preparation, microfilariae were isolated by density gradient centrifugation using HiSep™ and cultured in RPMI-1640 media. The microfilariae were filtered out at regular intervals and the media supernatant was collected. Excretory-secretory protein was obtained after concentrating the supernatant using Amicon Ultra filter with 3 kDa cut-off membrane. The concentration in the ESP preparation was estimated by Bradford's method using the Nanodrop 2000/2000c. Antigen preparations with a concentration of $>$ 200 mg/ml were utilised for coating the ELISA.

Standardization of plate ELISA

Checker board analysis was performed to derive optimum concentrations of ESP and optimum dilution of sera for plate ELISA. The maximum difference in optical density between known positive and known negative canine serum was observed with an antigen concentration of 300 ng and serum dilution of 1:300. Serum samples from 50 animals below the age of 4 months, which were PCR negative were tested in duplicates with serum dilutions using 300 ng of ESP. Cut off value was thus determined from the following formula.

$$\text{Cut off titre} = \text{Mean Optical Density} + 3\ (\text{Standard Deviation})$$

The standard deviation and mean optical density were found to be 0.1826 and 1.9280 respectively. The cut off titre was derived to be 2.4759.

The test proper was performed using samples which were collected from microfilaria positive animals (n = 50) and negative samples (n = 50). The OD values for the positive samples were observed to be higher than the cut off value. All serum samples from the negative animals were lower than the cut off values. Sensitivity and specificity were calculated in comparison with blood smear examination according to the formula mentioned in below.

The sensitivity was found to be 84 per cent and the specificity was found to be 100 per cent.

Comparison of results of blood film examination with indirect plate ELISA

For comparison of the Indirect ELISA with blood film examination, Chi square test we used. The results of the test are presented in Table 1. Indirect ELISA was found to be dependent on blood smear examination. Kappa value was found to be 0.840. This indicates high agreement in the detection between blood film examination and Indirect ELISA.

Discussion

The ELISA developed had a sensitivity of 84 per cent and the specificity of 100 per cent. Statistical analysis of blood smear examination with the Indirect EISA showed that the test developed had high agreement with blood smear examination, shown by Kappa value of 0.840. Hence, this ELISA may be considered for screening of dogs for brugian filariasis.

These findings agree with Kumari et al. (1994) and Chenthamarakshan et al. (1996). Chenthamarakshan et al. (1996) used ESP from *B. malayi* microfilariae and standardised an ELISA with sensitivity and specificity of 75 and 100 per cent respectively. Kumari et al. (1994) found a specificity and sensitivity of 75 and 100 per cent by using Bm12, recombinant protein from ESP of adult *B. malayi.*

The Indirect ELISA developed using crude protein from *B. malayi* by Rahmah et al. (1998) detected antifilarial IgG4 in a higher per cent of cases when compared to the Giemsa examination of thick blood smears thus reporting higher sensitivity.

A lower sensitivity in the current study might be due to the down regulation of antibodies which play a role in the higher loads microfilaremia and antigen loads and was seen in about 10 per cent of the microfilaria positive cases (Marley et al. 1995). However Noordin et al. (2003) found no correlation between the microfilaria count and the antibody titre. Another possibility was the lower levels of titre noticed in patients who had been treated with DEC. Since previous history of administration of microfilaricidal drugs was not known a definite correlation cannot be proven. Immunocompromised patients may also show lower antibody titres.

A wider population of dogs infected with *Dirofilaria* spp., *Acanthochelionema* spp. and endemic normal dogs with and without soil borne helminthic infections were not analysed in the current study. Thus after assessing this ELISA in a larger population of dogs along with blood

Table 1 Comparison of results of blood film examination with that of indirect ELISA

Indirect ELISA	Blood film examination				Total	
	Negative		Positive			
	Count	Per cent	Count	Per cent	Count	Per cent
Negative	50	100.0	8	16.0	58	58.0
Positive	0	0.0	42	84.0	42	42.0
Total	50	100.0	50	100.0	100	100.0

Chi square value = 72.414**; $p < 0.001$

Kappa value = 0.840

smear examination, the current procedure might be considered as an epidemiological tool.

Conclusion

This is the first report of successful isolation excretory/secretory protein from *Brugia malayi* microfilariae separated from canine blood and the standardization of ELISA using the antigen. The ELISA developed using excretory/secretory protein can be used to diagnose brugian filariasis in canines. Statistical analysis revealed high agreement between the blood smear examination and Indirect ELISA indicating its potential use as a diagnostic tool.

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Author contributions PVS: MVSc scholar performed the research. DC: Major guide for the MVSc Research who formulated the research work and gave instructions in performing the research and helped in preparing the manuscript. UNP: Minor guide for the MVSc research and Head of the Department who provided all guidance for the research. NMU: Minor Guide for the MVSc Research and guided for the research. BL: Minor Guide for the MVSc Research and guided for ELISA standardization. CS: Helped in analyzing the data statistically.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The project is formulated following the guidelines of Institutional Animal Ethics Committee and got approval from Faculty Research Committee with code number Ad/9/96/MVM/2015/CM. It is certified that all biomaterials required for the research were collected with prior written consent from the owners of the animals.

Informed consent The article is a part of M.V.Sc. thesis submitted to Kerala Veterinary and Animal Sciences University and submitted with consent from all authors.

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