

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.908.154>

Sero-prevalence of *Dirofilaria repens* Infection in Dogs by Indirect-ELISA using Microfilarial and Adult Worm Antigen

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ABSTRACT

Keywords

Sero-prevalence, *D. repens*, Dogs, Indirect-ELISA, Shivamogga, Mangalore

Article Info

Accepted:
15 July 2020
Available Online:
10 August 2020

Dirofilariosis is vector borne parasitic diseases of dogs caused by filarial nematodes of the genus *Dirofilaria*, which includes more than 40 different species. Among them *D. immitis* causes heartworm disease, whereas *D. repens* causes subcutaneous dirofilariosis in dogs. A study was conducted to determine seroprevalence of *D. repens* in dog from Shivamogga and Mangalore regions of Karnataka using Indirect-Enzyme Linked Immuno Sorbent Assay (Indirect-ELISA). A total of 315 dog blood samples were collected for detection of microfilaria by modified Knott's method and serum samples of the same dogs were screened for antibodies of *D. repens* by Indirect-ELISA using microfilarial and adult worm antigen. Among 315 serum samples screened, 220 and 183 were showed antibodies to microfilarial and adult worm antigen respectively. The seroprevalence of *D. repens* in dogs recorded in the present study using microfilarial and adult worm antigen was 69.84 and 58.09 per cent respectively. The sensitivity and specificity of Indirect ELISA with microfilarial antigen was found 100 and 64.09 per cent whereas, with adult worm antigen 100 and 72 per cent respectively. This form the first report that, the microfilarial and adult worm antigen of *D. repens* was used for the detection antibodies of *D. repens* in dog by indirect- ELISA from Karnataka. The present study showed that, the microfilarial antigen was found more sensitive compared to adult worm antigen in detection antibodies of *D. repens* in dog. The result indicates that, the microfilarial antigen can be effectively used for seroprevalence study at field level for large population.

Introduction

Dirofilariosis is vector borne nematode infection of dogs caused by several species of filarid nematodes belonging to the superfamily Filarioidea and family Onchocercidae. Nine filarial nematodes known to infect dogs worldwide includes *Acanthocheilonema* *reconditum*,

Acanthocheilonema dracunculoides, *Brugia malayi*, *Brugia pahangi*, *Brugia ceylonensis*, *Brugia patei*, *Cercopithifilaria grassii*, *Dirofilaria immitis* and *Dirofilaria repens*. Among these *D. immitis* is the most pathogenic canine filarid nematode, causes heartworm disease in dogs whereas, *D. repens* responsible for subcutaneous dirofilariosis. Even though *D. repens* is considered as less

pathogenic in dogs, the ability to infect humans makes it as zoonotic important parasite. Canine filariasis is reported from many countries across the world including India. Many filarial species were reported from different parts of India including Kerala, Tamil Nadu, Karnataka, Orissa, West Bengal, etc.

Diagnosis of dirofilariosis in dogs is mainly performed by conventional tests viz., wet blood film method, modified Knott's technique, Giemsa's staining, histochemical staining technique, citrate-saponin-acid method and quantitative buffy coat technique for detection of microfilariae in blood. Among conventional tests, modified Knott's test is currently considered as the gold standard for detection of circulating microfilariae in the blood (Di Cesare *et al.*, 2013). The serological tests like ELISA used for detection of circulating antigen or antibody and molecular techniques (PCR) for species identification. Apart from these diagnostic methods, isolation of adult worms at necropsy or from skin nodules can be used for morphological studies.

Presently serological diagnosis of heartworm infection by detection of circulating antigen in serum, plasma or blood samples of dogs using commercial antigen test kits is recommended for screening. This test detects a glycoprotein secreted by adult female worm and is the most sensitive diagnostic method currently available. At present, there are no commercial diagnostic test kits available for detection of *D. repens*. Some researchers conducted ELISA with *D. immitis* adult worm excretory/secretory antigen and *D. repens/D. immitis* adult worm somatic antigens for the detection of specific antibodies in dogs with filarial infections (Cancrini *et al.*, 2000; Joekel *et al.*, 2017). The present study was undertaken to determine seroprevalence of *D. repens* in dogs from Shivamogga and Mangalore regions of Karnataka using

microfilarial and adult worm antigen of *D. repens* by Indirect-ELISA.

Materials and Methods

Collection of samples

In the present study, a total of 315 blood samples were collected in vacutainer with and without anti-coagulant from dogs presented to Teaching Veterinary Clinical Complex, Veterinary College, Shivamogga and private clinics in Mangaluru as well as dogs in non-governmental organization at Mangaluru. The blood samples collected with anti-coagulant were used for detection of microfilaria by modified Knott's method.

Field Serum samples

In the laboratory, blood samples collected without anti-coagulant were used for separation of serum by centrifugation, aliquoted and stored in the deep freezer at -20°C until further use.

Positive and negative sera

The serum samples of dogs with adult worms of *D. repens* and microfilaria positive are used as true positive sera whereas, two-week old puppy serum was used as negative controls.

Adult worm antigen

The adult worms were collected from the skin nodules of dogs during sterilization at non-governmental organization, Mangaluru in Phosphate Buffer Saline (PBS, pH-7.2) and were washed three times thoroughly in Hank's balanced salt solution. Then, the worms were stored in PBS and deep frozen. The contents were repeatedly frozen and thawed four times and were triturated using a glass mortar and pestle. Then disrupted by sonication using ultrasonicator (Sonirep 150,

Sanyo Gallenkamp PLC, UK) for 4 min on ice at 400 W in pulsed mode. The suspension was centrifuged at 10000 rpm for 30 min in a refrigerated centrifuge (4°C) (Superspin). The supernatant was collected and used as the soluble antigen. The purification and concentration of antigen was done by dialysis. Then protease inhibitor- PMSF (Sigma, USA) was added at concentration of 2 µl/ml of antigen and aliquoted, stored at -20°C till further use. The identification of the worms was done based on the morphological characters described (Soulsby 1982; Bowmann 2009).

Microfilarial antigen

Isolation of microfilaria from blood was performed as per the procedure described by Franks and Stoll (1945) with slight modification. Approximately 20-40 ml of blood was collected from microfilaraemic dogs in centrifuge tube containing 5% sodium citrate. The citrated blood was centrifuged for 30 min at 2500 rpm and supernatant was removed. The packed red cell and microfilarial mass were brought to original blood volume with a 4:1 saline citrate solution. Approximately one ml of 15% solution of saponin in physiological saline was added for each 15 ml of original blood volume. Then the hemolyzed blood was centrifuged for 30 min at 2500 rpm and supernatant was discarded. The stroma-microfilaria sediment was washed two to three times with saline-citrate solution to remove as much of saponin as possible. The supernatants were discarded and microfilariae caught in the stroma sediment were released by adding saline-citrate solution, shaking vigorously and centrifuging at high speed for 30-60 sec. This process was repeated for several times and concentrated microfilaria was stored at -20°C until further use. Then the microfilarial antigen was prepared by sonication as per Schucan *et al.*, (2012).

Estimation of protein concentration

The protein concentration of both microfilarial and adult worm antigen was estimated by Bradford method (Bradford, 1976) by using protein estimation kit obtained from Bangalore Genei Co., Bangalore.

Indirect ELISA (Enzyme-linked immunosorbent assay)

Indirect ELISA was performed by following the procedure of Schucan *et al.*, (2012) using microfilarial antigen and adult worm antigen. The working dilutions of conjugate, antigen and test sera were determined by checkerboard titrations. The cut off value was calculated by taking mean absorbance values of known negative sera plus three standard deviation. Any serum with OD values above the cut off value was regarded as positive.

The flat bottom polystyrene 96 well ELISA plate was coated (100 µl/well) either with microfilarial antigen (5µg/ml) or adult worm antigen (5µg/ml) diluted in coating buffer in duplicates. The plate was incubated at 4°C overnight and washed thrice with washing buffer. The plates were incubated at 37°C for one hour after adding 100 µl of blocking buffer (5% skimmed milk powder with PBS Tween-20) and washed thrice with PBS containing Tween-20. The positive serum (1:50 dilution) with blocking buffer was added to all wells and incubated for one hour at 37°C. The plates were washed four times with washing buffer and 100 µl of 1:2,500 diluted anti-dog conjugate was added and incubated as above. The plates were washed five times with washing buffer. Then 100 µl of OPD substrate chromogen working solution was added and color reaction was monitored in dark place. The reaction was stopped by adding 50µl of 2M H₂SO₄. The absorbance values were read in a Multiscan plus P (Lab systems) ELISA reader at 450

nm. Positive control and negative control was included in the assay in duplicate.

Sensitivity and specificity of Indirect-ELISA

The sensitivity and specificity of ELISA was calculated by the following formula:

$$\text{Sensitivity: } \frac{\text{True Positive}}{\text{True positive} + \text{false negative}} \times 100$$

$$\text{Specificity: } \frac{\text{True negative}}{\text{True negative} + \text{false positive}} \times 100$$

Results and Discussion

In the present study, a total of 315 blood samples were screened for detection of microfilaria by modified Knott's method. Among, 315 samples screened, 93 were found positive for microfilaria (Fig. 1). The species of microfilaria was identified based on the morphology and micrometry by modified Knott's method. Morphologically, the microfilariae were unsheathed, with blunt head and a tapering tail (Fig. 2). The biometrical studies revealed that, the length of the microfilaria were in the range of 298 to 312 μm whereas, the width in the range of 8.6 to 10.5 μm . The adult worms recovered from the subcutaneous tissue of dogs were identified as *D. repens*. Morphologically, adult female worms were long, hind part was tapered and blunt, whereas males were short and hind part was coiled and pointed (Fig. 3). The outermost layer of the adult worm showed well-developed thick multilayered cuticular ridges followed by transverse smooth muscles striations. The micrometry of female worm measured 110 to 160 mm in length and 4.3 to 6.1 mm in thick whereas male worms measured 48 to 69 mm in length and 3.6 to 4.3 mm diameter.

In the present study, the protein concentration of the microfilarial (Fig. 3) and adult worm

antigen (Fig. 4) was estimated by Bradford method and found 825 $\mu\text{g/ml}$ and 875 $\mu\text{g/ml}$ of antigen respectively. By checkerboard assay method the working dilutions of conjugate, microfilarial antigen and positive serum were standardized as 1:2500, 5 $\mu\text{g/well}$ and 1:50 respectively, whereas working dilutions of conjugate, adult worm antigen and positive serum as 1:2500, 5 $\mu\text{g/well}$ and 1:50 respectively. The result of the preliminary assay performed on negative sera from 10 dogs yielded a mean background absorbance value (x) of 0.230 and 0.236 and a standard deviation of 0.029 and 0.028 for microfilarial and adult worm antigen respectively. In the present study, the cut off OD value for microfilarial antigen was calculated as 0.319 and for adult worm antigen as 0.321 (Mean + 3 SD).

The results of indirect ELISA to detect specific antibodies against microfilarial antigen of *D. repens* are presented in the Table 1. Out of 315 serum samples screened for the presence of antibodies against *D. repens* using microfilarial antigen in dogs, 220 samples showed positive reaction (Fig. 5) with a seroprevalence of 69.84 per cent. The OD values of positive serum were in the range of 0.328 to 0.847.

The results of indirect ELISA to detect specific antibodies against adult worm antigen of *D. repens* are presented in Table 1. Out of 315 serum samples screened for presence of antibodies against *D. repens* using adult worm antigen in dogs, 183 samples were found positive (Fig. 6) with a seroprevalence of 58.09 per cent. The OD values of positive serum were in the range of 0.323 to 0.809. The sensitivity and specificity of Indirect ELISA with microfilarial antigen was found 100 and 64.09 per cent whereas, with adult worm antigen 100 and 72 per cent respectively (Table 2). The statistical analysis by Chi-square test revealed significant difference between the two antigens ($P < 0.01$).

Table.1 Seroprevalence of *D. repens* infection in dogs by indirect ELISA

Type of antigen	No. of dogs screened	No. of dogs positive	Percent positive	X ² value and significance
Microfilarial antigen	315	220	69.84	9.428**
Adult worm antigen	315	183	58.09	

Note: ** - Significant at p<0.01

Table.2 Sensitivity and specificity of Indirect ELISA in detection of *D. repens* infection in dogs using microfilarial and adult worm antigen

	Sensitivity (%)	Specificity (%)
Microfilarial antigen	100	64.09
Adult worm antigen	100	72.00

Fig.1 Microfilariae of *D. repens* by modified Knott's method (100X)

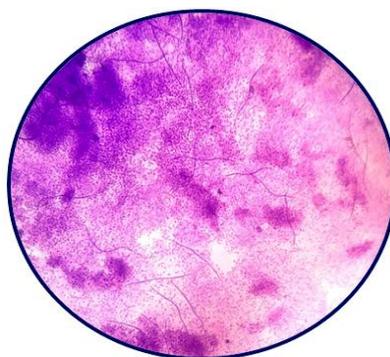


Fig.2 Microfilaria of *D. repens* in modified Knott's method: unsheathed, (a) blunt head (b) tapering tail (400X)

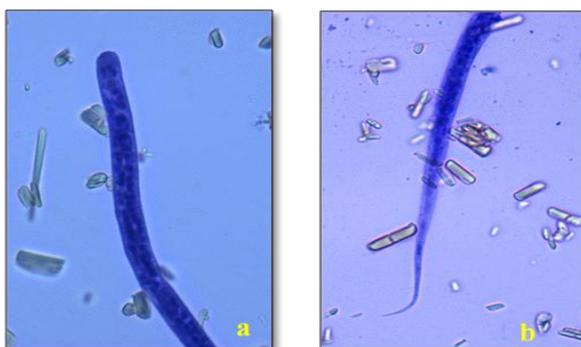


Fig.3 Concentrated *D. repens* microfilariae

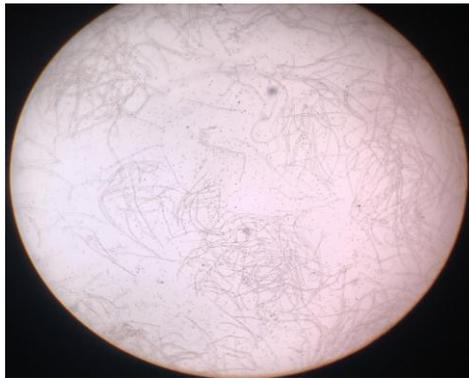


Fig.4 Adult worm of *D. repens*: Male (a) Female (b)



Fig.5 Indirect ELISA with microfilarial antigen for detection of antibodies against *D. repens* in field sera along with positive and negative control

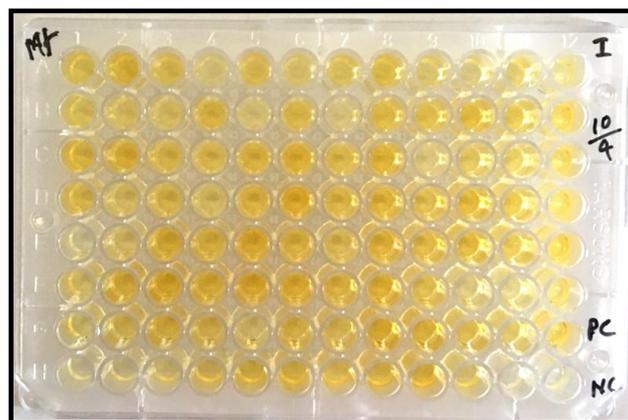


Fig.6 Indirect ELISA with adult worm antigen for detection of antibodies against *D. repens* in field sera along with positive and negative control



In the present study, out of 315 blood samples screened by modified Knott's method, 93 (29.52 %) were found positive for microfilaria. The microfilaria and adult worm recovered from the dog were identified as *Dirofilaria repens* based on morphological characters and micrometry by modified Knott's method as per the description of Soulsby (2005) and Bowman (2014). Magnis *et al.*, (2013) reported that, Knott's test enables to clearly distinguish between *D. immitis*, *D. repens* and *Acanthocheilonema* spp. based on morphological and morphometric studies. Gunathilaka *et al.*, (2017) identified *Dirofilaria repens* adult worm extracted from the subcutaneous nodule of human with a similar morphological character from Sri Lanka.

Microfilariae detecting conventional diagnostic techniques are not able to detect occult infections or low microfilaremic infections. These infections arise due to several causes including low parasite burdens, prepatent infection by young adults, unisexual infection, drug induced sterility of adults or immune-mediated clearance of microfilariae (Rawlings *et al.*, 1982). Enzyme-linked immunosorbent assays can be used for serological diagnosis of filarial infections,

which can even detect occult or hidden or past infections. However, the samples positive by microfilarial detection, but negative for antigen detection ELISA kits could be attributed to immune-mediated clearance of antigen-antibody complexes (Magi *et al.*, 2012).

Several commercial antigen test kits are available for serological diagnosis of *D. immitis* infection by detection of circulating antigens from adult female worm in serum, plasma or blood samples of dogs. These ELISA tests for diagnosis of *D. immitis* infection are considered highly specific, as cross reactivity with other canine filarid nematodes such as *D. repens* and *Dipetalonema* spp. does not occur (Venco and Vezzoni, 2001). The sensitivity of test is actually very high, but false negative results occur in prepatent period or very light infections. However, several studies (Martini *et al.*, 1996; Klotins *et al.*, 2000; Stone and Mackereth, 2003 and Atkins, 2003) reported that, the commercial serological kits have low sensitivity when parasitic burdens are low (one to five *D. immitis* adult females), when the worms show low fertility and also could be due to the presence of microfilariae during 1–3 years after the dead of adult females.

Antigen and microfilarial tests will give a false negative result if the infection is prepatent (adult female worms are not yet sexually mature and producing microfilariae) or unisexual with only male worms present. Antibody tests are more useful in pre-patent infections but reveal false positive result if worms have died but antibody levels have not yet declined below the limit of detection (Levy *et al.*, 2003). Antibody tests will also give a false negative result if the patient is infected but has not yet developed sufficient levels of antibody (Berdoulay *et al.*, 2004). The combination of antigen and antibody detection tests increases both sensitivity and specificity (Snyder *et al.*, 2000). Other factors affecting test performance includes operator skill, test performance over time and sample handling and quality (Greiner and Gardner, 2000).

Presently, there are no commercial serological test kits are available for detection of *D. repens*. However, some researchers have conducted ELISA for the detection of specific antibodies in dogs using *D. immitis* adult worm excretory/secretory antigen and *D. repens/ D. immitis* adult worm somatic antigens (Cancrini *et al.*, 2000; Simsek *et al.*, 2011; Tasic *et al.*, 2012 and Joekel *et al.*, 2017).

Many researchers across world were reported seroprevalence of *D. immitis* infection in dogs using commercial ELISA test kits in the range of 1.3 - 42.8 per cent (Alves *et al.*, 1999; Svobodova *et al.*, 2005; Simsek *et al.*, 2008; Ben Mahdi and Mohamed, 2009; Rapti and Rehbein, 2010; Ionica *et al.*, 2014; Girdan *et al.*, 2015; Diakou *et al.*, 2016; Ferreira *et al.*, 2017 and Atas *et al.*, 2018). Whereas, Borthakur *et al.*, (2015) reported 18.03 per cent seroprevalence of *D. immitis* infection in dogs from north-eastern states of India by using commercial ELISA test kit.

Among 315 serum samples screened by Indirect ELISA for detection of antibodies against *D. repens*, 220 and 183 were showed antibodies to microfilarial and adult worm antigen respectively. The seroprevalence of microfilariosis in dogs recorded in the present study using microfilarial and adult worm antigen was 69.84 and 58.09 per cent respectively. Cancrini *et al.*, (2000) reported 9.64 per cent seroprevalence of *D. repens* infection in dogs, whereas, Tasic *et al.*, (2012) reported 55.9 per cent seroprevalence of *D. repens* infection in dogs, both were used adult worm somatic antigen for ELISA.

The variations in the rate of seroprevalence might be due to the variation in the type of antigen used and procedure followed for preparation of antigen, difference in diagnostic tests, study population and infection status of the dogs. The seroprevalence of *D. repens* detected by indirect ELISA in the present study was higher compare to QBC, probably due to detection of occult or past infections.

Razi Jalali *et al.*, (2010) reported that, the sensitivity and specificity of rapid heart worm antigen test kits in detection of *D. immitis* antigen was 98.5 and 100% respectively whereas, Joekel *et al.*, (2017) found sensitivity and specificity of the monoclonal antibody based sandwich-ELISA assay for detection of antibodies against *D. repens* was 100 and 98.6 per cent respectively. The sensitivity of the indirect ELISA used in the present study was 100 per cent with both microfilarial antigen and adult worm antigen, whereas specificity was 64.09 and 72 per cent with microfilarial antigen and adult worm antigen respectively. Low specificity of indirect ELISA used in the present study compare to previous assays, indicate the increase in false positive results due to homologous helminth interspecies epitopes leading to cross-reactions (Joekel *et al.*, 2017).

In conclusion this is the first report, where the microfilarial and adult worm antigens of *D. repens* used to study the seroprevalence of *D. repens* by Indirect-ELISA in dogs from Shivmogga and Mangaluru regions of Karnataka. It was observed that, microfilarial antigen was more sensitive compared to adult worm antigen to study the sero-prevalence of *D. repens* in dogs. Hence, the microfilarial antigen can be effectively used for detection of *D. repens* antibodies in dogs at field level for sero-prevalence study in large population.

Acknowledgement

The Authors are grateful to Department of Veterinary Medicine, Veterinary College Bangalore for providing financial assistance and Department of Veterinary Parasitology, Veterinary College, Shimoga for providing laboratory facility to conduct the present study. The authors are also thankful to Dr. Yashawi Naravi for their co-operation and help during the present study.

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How to cite this article:

Malatesh, D. S., C. Ansar Kamran, K. J. Ananda and Manjunath Reddy, G. B. 2020. Sero-prevalence of *D. repens* Infection in Dogs by Indirect-ELISA using Microfiarial and Adult Worm Antigen *Int.J.Curr.Microbiol.App.Sci.* 9(08): 1355-1365.

doi: <https://doi.org/10.20546/ijcmas.2020.908.154>