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Zoonotic canine dirofilariasis; molecular detection in dogs infected by *Dirofilaria repens*

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Abstract
Canine subcutaneous diroflariosis has been evinced recently with an increasing number of cases in the Orathanadu taluk Thanjavur District Tamilnadu and however there are no published data on the presence and prevalence of *Dirofilaria repens* in dogs of this region in the State. The present study provides information about the prevalence of Dirofilaria in dogs and molecular means of PCR detection of *D.repens* from subcutaneous tissues embedded with the worm in affected dogs of this region. Of the127 dogs sampled, 77.9% (n = 99) were positive for *D. repens* circulating antigen and found positive by PCR for ITS2 and PCR was the most sensitive and specific method, capable of detecting *D.repens* infections. To the authors' knowledge, this is the frst report of *D. repens* infection in dogs in Cauvery Deta Region identified by PCR for Cox1 gene being the main etiological agent of diroflariosis in Thanjavur District.

Keywords: *Dirofilaria repens*, dog, Cox1 gene, PCR

Introduction
Dirofilariosis is a potentially zoonotic filarial parasitic disease, present in several parts of the world, transmitted mainly by mosquito vectors. The species *Dirofilaria immitis* and *Dirofilaria repens* (Filarioidea, Onchocercidae) are widely present in the Asian and Mediterranean basin and are the causative agents of cardiopulmonary and subcutaneous dirofilariosis respectively. Both nematodes are transmitted by mosquito species of the family Culicidae and can infect domestic and wild canids and felids, causing severe pathological effects McCall *et al*.,(2008) [1]. Canine subcutaneous diroflariosis (*D. repens*) is often considered asymptomatic, although in some cases the parasites cause subcutaneous nodules, while circulating microflariae cause dermatological signs such as pruritus, erythema, alopecia, difused dermatitis and itching Tarello (2002) [2]. In the last decade, molecular identification techniques have been developed for specific diagnosis of various species of Dirofilaria Tse *et al*., (2010) [3]. *Dirofilaria immitis* is considered the most virulent filarial species in dogs, as the long-lived adult worms reside in the right ventricle and pulmonary artery, leading to pulmonary hypertension, congestive heart failure and even death but *D. repens* adult forms live in subcutaneous tissue, where they cause dermatological problems, such as multifocal nodular and prurigo papularis dermatitis. Moreover, both species may also infect humans. *Dirofilaria repens* adult/pre-adult stages may induce subcutaneous and ocular lesions Pampiglione and Rivasi (2000) [4]. The aim of the present study was to identify the Dirofilaria species currently circulating in dogs through an optimised reliable and highly sensitive species-specific PCR assay forearly detection and associated haematological changes of *D. repens* in canines of various breeds.

Materials and methods
Sample collection
During January 2019-December 2019, an investigation was carried out for a period of about a year and 127 dogs comprising of predominantly Labrador (pet dogs (45) and nondescript dogs (41) of both sexes and various age groups and Dobermann breed (22) and Pomerian (19) were studied from nearby regional veterinary dispensaries who brought samples to Veterinary University Disease Diagnosis Laboratory, VCRI, Orathanadu College, Tamilnadu. The suspected dogs after surgical operations were screened for dirofilariasis of presence of adult worms of *D.repens* and identified.

Morphological identification of *D. repens*
The collected adult nematodes were measured and morphologically studied using light microscopy. The parasites were identified by an evaluation of the macroscopic and microscopic characteristics.

About 1 cm of the nematode cephalic and caudal end was prepared with 50% glycerol for transparent slides. The middle parts of the nematodes were used for molecular identification. The length and the distance between the oral opening and vulva (for females) or the length of the left and right spicule (for males) of the nematodes were measured for morphological nematode identification Manfredi *et al*., (2007) [5].

DNA isolation

DNA was extracted from crushed adult worms after morphological identification amine tetraacetic acid) was incubated with 600 µl CTAB buffer and 0.2 mg proteinase K (Bioline, London, UK) at 56 °C for 2 h, with agitation. DNA precipitation was done with 0.6 ml absolute ethanol and the pellet hydrated in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0). DNA samples were stored at -20 °C until further use. Deionised water was used as a PCR negative control. DNA amplification the ribosomal internal transcribed spacer (ITS) region was amplified using two different PCR protocols for molecular screening of canine filarial species. The internal transcribed spacer 1 (ITS1) region was amplified using a semi-nested PCR as described by Nuchprayoon *et al*. (2003) [6]. Briefly, primers FL1-F and FL2-R were used in a first-round PCR to amplify the entire ITS region, and primers FL1-F and Di5.8S 660-R in a second-round PCR to amplify the ITS1 region, with expected amplification fragment size of 650 bp for *D. repens*.

Results and Discussion

Out of 127 dogs seventy eight dogs underwent the castration and other surgical operations were found with *Dirofilaria repens* in the subcutaneous tissue.Morphologically the adult worms were identified as *Dirofilaria repens* (Fig 1&2). On molecular characterisation all the ninety nine samples proved to be positive for Cox 1 gene of *D.repens* by PCR method of identification(Fig 2) which is in accordance with the laboratory findings of Sabunas *et al*(2003) [7].

Fig 1: Adult worms noticed during castration operation

Fig 2: Head end (conical) of *D. repens* under 10x microscope

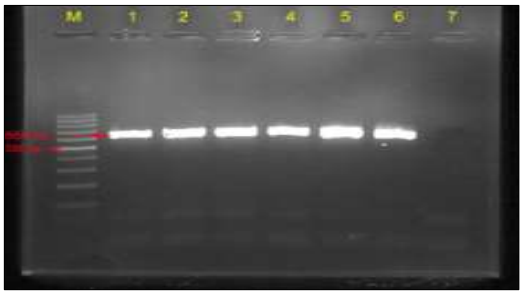

Fig 3: Agarose gel electrophoresis showing 650 bp PCR product of cox 1 gene amplified from sedimented *D. repens*

The case of the dog described here presented all such features, but differed under many aspects from the several other cases of subcutaneous dirofilariasis already reported in other regions of the world such as Portugal (Sabunas *et al* 2003[7] and Italy Genchi *et al*. 2019 [8].

In conclusion, The research on molecular means of characterisation carried out in here suggest that *D. repens* can be diagnosed earlier and can be used as a rapid technique for screening of filarial infections in dogs and in future can be used for differentiation and adoption of regulations in controlling dirofilariasis in canines than the conventional means of diagnosisng from other concurrent blood microfilariae.

The dogs were treated after a two days of rest, with macrofilaricides (Melarsomine, 2,5 mg/Kg., i.m., b.i.d.) and a microfilaricide drug (Ivermectine, 50 µg/Kg, s.c.) was administered 10 days later, when complete remission of the syndrome could be observed. Canine Dirofilariasis from dogs are the most important source for human transmission of diroflariosis McCall *et al*., (2008) [1]. There should be stricter rules around the use of preventive measures and regulations for free-ranging dogs in future.

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