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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2015.11.010>Human ocular dirofilariasis due to *Dirofilaria repens* in Sri Lanka

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ABSTRACT

Objective: To identify worms obtained from patients with eye lesions and to describe the demographic factors of patients with ocular dirofilariasis.**Methods:** A retrospective descriptive study was conducted in 31 worm samples from 30 patients referred by consultant ophthalmologists between 2006 and February 2014. Data on age, sex and site of the lesion were ascertained from the details given in the referral letters. Morphological identification of the worm was based on the maximum width, length and appearance of the cuticle. The sex of the worm was determined by the width, length and presence or absence of vulva opening. PCR was performed using *Dirofilaria repens* specific primers to confirm the species of worms which couldnot be identified morphologically.**Results:** Most of the patients belonged to the age group of 40–49 years (mean age = 42 years). Majority of them were females (70%). Subconjunctival lesions were the most frequent presentation, while the rest ($n = 4$) were found on eyelids. Female worms were extracted from 18 cases, and 11 had male worms. One individual had both male and female worms in a single nodule. Adults were the most commonly affected. This pattern was different from the previous studies in Sri Lanka where the most common age group affected was younger than 9 years old.**Conclusions:** The present study showed a considerably high incidence of ocular dirofilariasis, stressing the importance of implementing preventive measures to reduce the transmission of this zoonotic filarial disease.

1. Introduction

Human dirofilariasis is a zoonotic disease caused by infection with several species of nematodes belonging to the genus *Dirofilaria*. The most common *Dirofilaria* species causing human infections are *Dirofilaria repens* (*D. repens*) and *Dirofilaria immitis* [1]. *D. repens* is commonly found in subcutaneous tissues of dogs, foxes and cats, while *Dirofilaria immitis* inhabits right ventricles and pulmonary arteries of dogs and cats [2]. Other non-canine associated species that occasionally cause human infections include *Dirofilaria tenuis* (from raccoons), *Dirofilaria ursi* (from bears), *Dirofilaria subdermata* (from porcupines) and *Dirofilaria striata* (from bobcats) [1,3–5]. Dirofilariasis is typically a disease of animals, which can also be transmitted

to humans by zoonophilic species of mosquitoes of the genera *Anopheles*, *Culex*, *Armigeres* and *Aedes* [6]. Mosquitoes obtain microfilaria from an infected host during a blood meal. Microfilaria develops into the third stage infective larva in malpighian tubules and migrates to proboscis through body cavity of the mosquito [2]. When this mosquito feeds on a dog, human or other hosts, it transmits the infective larvae into blood stream of the host. However, worms fail to reach maturity while residing in human body. Human infection usually presents with a parasite nodule [7]. Dirofilariasis is most commonly associated with subcutaneous and ocular lesions and is increasingly reported as aberrant migration of worms in humans worldwide [8,9].

D. repens can infect various parts of human body including eyes, lungs, soft tissues (including breast), brain, liver, intestine, lymphatic glands, and muscles [10,11]. The diagnosis of human dirofilariasis relies mainly on morphological features of the worm [1]. *Dirofilaria* is characterized by a relatively large size, thick cuticle, and prominent musculature with muscle cells extending far into body cavity [12]. Different *Dirofilaria*

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species can be distinguished by their size, thickness of cuticle, and presence or absence of longitudinal ridges [13]. In some cases, identification of *Dirofilaria* species based only on morphology is not possible. Therefore, the use of molecular methods like PCR is necessary for the effective identification of specific species [14]. Nuclear and mitochondrial genes are useful molecular markers to identify helminth species, and the latter genes have been frequently used to identify *Dirofilaria* species [15,16]. *Dirofilaria* species responsible for human disease vary according to geographical location. Human infections are most commonly due to *D. repens* in Europe and Asia, while in North America it is due to *Dirofilaria immitis* [3,4]. Endemic foci are seen in Southern and Eastern Europe, Asia Minor, Central Asia and Sri Lanka [17,18]. The present study was carried out to identify the worms obtained from patients with eye lesions and to describe the demographic factors of patients with ocular dirofilariasis.

2. Materials and methods

2.1. Case record

A retrospective descriptive study was conducted using samples of worms from 30 patients referred by consultant ophthalmologists between 2006 and February 2014. A total of 31 worm specimens extracted from ocular nodules in the conjunctiva, orbital region and eye lid were included in this study. Of these, 29 specimens were single worm nodules while one nodule had two worms. There were 26 intact worms and 5 fragmented worms. Species identification was performed at the Department of Parasitology, Faculty of Medicine, University of Peradeniya. Data on age and sex of the patients and site of the lesion were ascertained from details given in the referral letters.

2.2. Species identification

The samples were preserved in 70% (v/v) ethanol. The worms were identified using morphological keys published by Levine [19]. Identification of the worm was based on maximum width, length and appearance of the cuticle. The length and width of the worms were measured using an ocular micrometer of optical microscope at low (4×) magnification. A clearing agent, lacto phenol, was used to mount the worm material for observing the morphological features of worms. All worm samples were examined for the key markers; longitudinal ridges and vaginal openings using a range of (4×, 10× and 20×) magnifications of the optical microscope. All worm samples were processed for sex discrimination. Sex of the worm was discriminated by the width, length and distance between anterior end and genital openings. The worms that were 10–17 cm long and 460–650 μm wide with a vulva opening 1.15–1.62 mm from the anterior end were classified as female worms. The worms that were 5–7 cm long and 370–450 μm wide without the vulva opening were classified as male worms [19]. All intact worm samples ($n = 26$) were identified morphologically.

2.3. Genomic DNA isolation

Five worm fragments were subjected to PCR since the morphological identification was not possible. Prior to the DNA

isolation, 70% (v/v) ethanol was drained and adequate amount of worm material was left to air dry at room temperature. Genomic DNA was extracted from individual parasites using the Qiagen genomic DNA extraction kit.

2.4. PCR

Primers used in this study include: *DIR3* (5'-CCGGTA-GACCATGGCATTAT-3prime;) and *DIR4* (5'-CGGTCTTG-GACGTTTGGTTA-3') [20]. These primers are specific to a highly repetitive DNA element from the genome of the filarial nematode *D. repens* [21]. The PCR mixture contained DNA (5.0 μL), PCR buffer (10×, 2.5 μL), magnesium chloride (50 mmol/L, 2.0 μL), distilled water (10.0 μL), forward primer (10 pmol, 1.5 μL), reverse primer (10 pmol, 1.5 μL), dNTP (2.5 mmol/L, 2.0 μL) and *Taq*DNA polymerase (5 U/μL, 0.5 μL). The mixtures were amplified in 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 5 min in an automated thermal cycler (Amplifronyx, NyxTechnik, USA). The positive control used in the study was obtained from adult *D. repens* isolated from a dog. Standard precautions were taken to avoid PCR contamination, and no false-positive results were observed in the negative control.

2.5. Electrophoresis

The PCR products were run on a 1.5% agarose gel at 100 V and 250 mA for 45 min. The gel was observed under UV light (302 nm) and the images were captured using the software Alpha Imager mini.

3. Results

All the patients were from various clinics in the Central Province of Sri Lanka. The age range of the subjects affected was from 1 to 78 years with a mean age of 42 years. Majority of the patients belonged to the age group of 40–49 years (Figure 1). Seventy percent of the study populations were females.

The majority ($n = 18$) of worms were recovered from the subconjunctiva. The average length of female and male worms was (12.03 ± 1.85) cm and (6.23 ± 0.65) cm, and the average width of female and male worms was (504.41 ± 53.36) μm and (392.90 ± 29.75) μm, respectively. These results were in conformity with the measurements of *D. repens*. The sex of the worm was determined by measuring the length between anterior

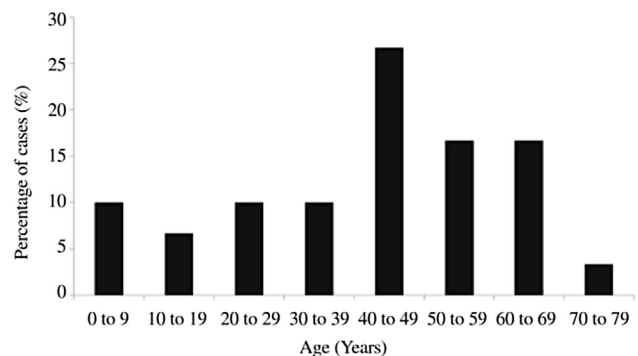


Figure 1. Distribution of human dirofilariasis categorized according to age groups of patients from 2006 to February 2014.

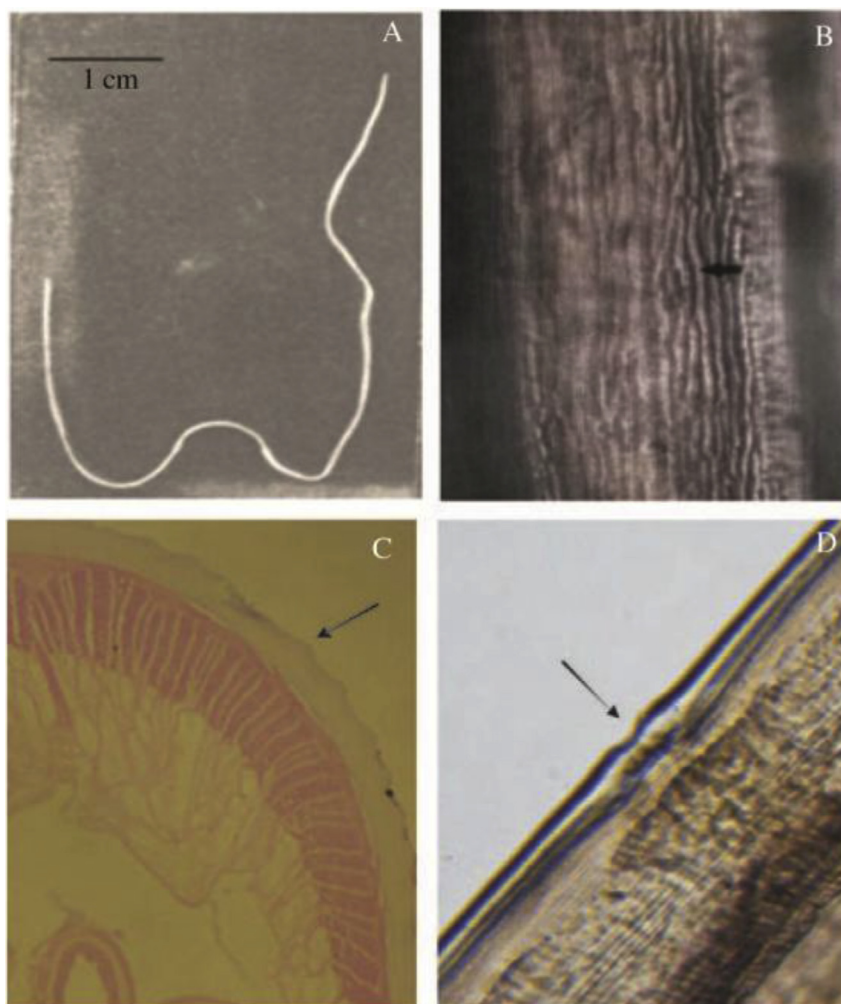


Figure 2. Morphological identification of *Dirofilaria* nematode.

(A): Macroscopic view of *D. repens* worm. (B): Microscopic view of outer cuticula with multiple longitudinal ridges (10×). (C): Cross-section of *D. repens* stained with H & E displaying cuticular ridges (10×) (D): Anterior end of female worm with the vulval opening (10×).

end and vulva. The female worm was extracted from 18 cases and 11 had a male worm. One individual had both male and female worms. Of the 31 worms, 26 were morphologically identified (Figure 2) as *D. repens* and the rest ($n = 5$) were

identified as *Dirofilaria* species. These five worm samples were confirmed as *D. repens* using PCR. Amplification was detected in the samples and positive control. The PCR amplified products yielded a band at 246 bp specific to *D. repens* (Figure 3). The negative control did not show any false positive result.

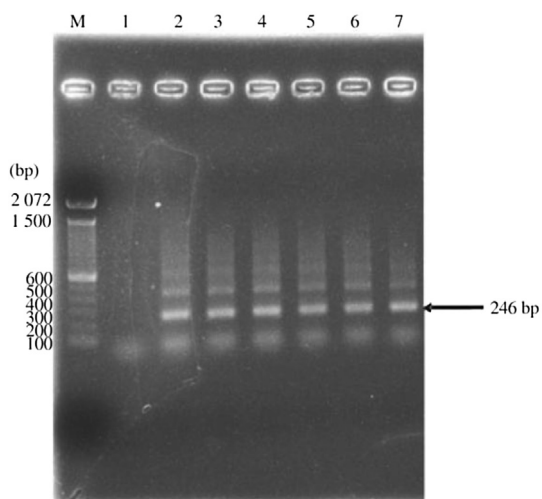


Figure 3. PCR amplification with primers DIR3 and DIR4.

Lane M: 100 bp DNA ladder; Lane 1: Negative control; Lane 2: Positive control; Lanes 3–7: Samples.

4. Discussion

The first human case of dirofilariasis in Sri Lanka was reported in 1962 [22]. Since then there has been an increasing number of cases, documenting the second largest collection of *D. repens* cases in the world [17]. The present study demonstrated a considerably high incidence of ocular lesions due to *D. repens*.

D. repens infects a number of different sites in human body. A review article based on data published between the years 1995–2000 concluded that majority (75.8%) of the cases had *Dirofilaria* infections in upper half of body, particularly ocular region which alone accounted for 30.5% of the total cases [17,18]. In ocular dirofilariasis, eye lesions usually involve periorbital, orbital and subconjunctival tissues [23,24]. Only a few intraocular lesions have been reported so far [25]. A majority ($n = 18$) of patients in the present study had subconjunctival lesions. Similarly, in several published ocular dirofilariasis

case studies, the majority of worms were located under conjunctiva [26,27].

In the present study, the infection was most common among individuals in the age group of 40–49 years, which is consistent with reports from European countries [17,28]. However, this does not follow the trend described previously in Sri Lanka in which the infection was most common among children under the age of 9 [29]. In this study, 70% of the infected patients were female which is in agreement with prior studies [17]. In the present study, majority of the cases had a female worm ($n = 18$) and this was concordant with results obtained in another study that reviewed 19 cases, of which 14 had a female worm [30]. In one case, both male and female worms were found in the subconjunctival lesion. Similarly, several studies have reported up to three worms dwelling in the same nodule [18,31–33].

A WHO project carried out in 1994 to determine the dog population in Sri Lanka reported a dog to human population ratio of 1:8 [34]. However, a survey carried out in 1999 has shown a sharp increase in dog population in urban areas which altered the dog to human population ratio to 1:4.6 within a 5-year period. A notable fact was that 20% of these dogs were stray [35]. Dirofilariasis is very common in dogs in Sri Lanka with a prevalence rate of 30%–60% [29]. In Sri Lanka, the mosquito species *Aedes aegypti*, *Armigeres subalbatus*, *Mansonia uniformis* and *Mansonia annulifera* have been shown to be efficient vectors for this parasite [29]. Thus, the risk of transmitting *Dirofilaria* is an increasing threat to human population in Sri Lanka. In conclusion, this study showed *D. repens* as the species responsible for ocular dirofilariasis in Sri Lanka, stressing the importance of implementing vector control and parasite control in dogs.

Conflict of interest statement

We declare that we have no conflict of interest.

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