

Dirofilaria repens in Vietnam: Detection of 10 Eye and Subcutaneous Tissue Infection Cases Identified by Morphology and Molecular Methods

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Abstract: From 2006 to 2010, hospitals in Hanoi treated 10 human patients for dirofilariasis. The worms were collected from parasitic places, and identification of the species was completed by morphology and molecular methods. Ten parasites were recovered either from the conjunctiva (n=9) or subcutaneous tissue (n=1). The parasites were 4.0-12.5 cm in length and 0.5-0.6 mm in width. Morphological observations suggested all parasites as *Dirofilaria repens*. Three of the 10 parasites (1 from subcutaneous tissue and 2 from eyes) were used for molecular confirmation of the species identification. A portion of the mitochondrial *cox1* (461 bp) was amplified and sequenced. Nucleotide and amino acid homologies were 95% and 99-100%, respectively, when compared with *D. repens* (Italian origin, GenBank AJ271614; DQ358814). This is the first report of eye dirofilariasis and the second report of subcutaneous tissue dirofilariasis due to *D. repens* in Vietnam.

Key words: *Dirofilaria repens*, case report, eye, subcutaneous tissue, PCR, mitochondrial *cox1*, Vietnam

INTRODUCTION

Human filariasis is caused by members of the Filaridae family, including species of *Dirofilaria*, *Brugia*, *Wuchereria*, *Onchocerca*, *Dipetalonema*, *Loa*, and *Meningonema*; it is transmitted to humans by various kinds of insect vectors [1]. Dirofilariasis is typically a disease of animals, which can also be easily transmitted to humans by mosquitoes of the genera *Anopheles*, *Culex*, and *Aedes* [2]. All of these mosquitoes are found in Vietnam. Of the 30 different species of *Dirofilaria*, *D. repens* and *D. immitis* are the 2 most common species that frequently infect humans [3]. Other *Dirofilaria* species have also been reported to infect Vietnamese carnivore species [4].

There have been over 1,000 cases of dirofilariasis, reported throughout the world, including 300 cases involving the lungs or viscera and over 800 cases involving the subcutaneous tissues or eyes [1]. Most of these were caused by *D. immitis* or *D.*

repens. *D. immitis* is a parasite of dogs and cats and it can occasionally become a causative agent of lung and subcutaneous dirofilariasis in humans. *D. repens* can also infect humans and is associated with diseases of various organs, including the conjunctiva, lungs, soft tissues (including the breast), brain, liver, intestine, lymphatic glands, and muscles [5,6].

In some cases, identification of *Dirofilaria* spp. based only on the morphology is difficult. Therefore, the use of molecular methods, such as PCR, is necessary for effective species identification [7]. Nuclear and mitochondrial genes are useful for the identification of helminth species, and especially the latter genes have been frequently used for identification of *Dirofilaria* spp. [8-10].

Given that there has been an increasing number of patients suffering from *D. repens* infection, further research is required on this newly emerging zoonotic disease as a public health threat in Vietnam. Accurate diagnosis, proper identification, and control measures are therefore needed to control human dirofilariasis in Vietnam.

CASE RECORD

During 2006 to 2010, a total of 9 patients with a swelling

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mass under their conjunctiva admitted to the National Eye Hospital (NEH), and a patient with a swelling in the subcutaneous tissue admitted to the Military Hospital 108. By surgery, live parasites were collected from these patients and species identification was tried. The total 10 patients, 27-77 years old, were from 4 provinces in the North Vietnam, including Hanoi City (4 patients), Ninh Binh province (3 patients), Ha Nam province (2 patients), and Hung Yen province (1 patient) (Table 1). Nine of them had similar symptoms, such as a painful, itchy, swollen, and tangible nodule in the eye; 6 cases involved the right eye and 3 involved the left. Another patient, 36-year-old, had a tumor (3×4 cm) in the left subcutaneous tissue, which appeared as a red nodule and was itchy. Surgical biopsies were performed on all patients and living parasites were recovered from each patient.

Parasites measured 4.0-12.5 cm in length and 0.5-0.6 mm in width. The worms were identified by the morphology, and pictures were taken (Fig.1). Among the worms, 3 were chosen (2 from the conjunctiva and 1 from the subcutaneous tissue), marked as GCA-VN1, GCH-VN2, and GCD-VN3, respectively, and analyzed by molecular methods.

Table 1. Information of worms collected from patients

Serial no. cases	Sex	Age (years)	Province	Parasitic place	Worm length (cm)
1	Female	50	Hanoi	Right conjunctiva	4.0
2	Male	47	Hanoi	Left conjunctiva	8.0
3	Female	27	Hanoi	Left conjunctiva	10.0
4	Male	49	Hanoi	Right conjunctiva	5.0
5	Male	77	Ninh Binh	Right conjunctiva	11.0
6	Female	60	Ninh Binh	Right conjunctiva	15.0
7	Female	55	Ninh Binh	Right conjunctiva	11.0
8	Female	50	Hung Yen	Right conjunctiva	10.0
9	Male	50	Ha Nam	Left conjunctiva	12.5
10	Male	36	Ha Nam	Left subcutaneous side	12.0

Identification of species

Parasites recovered from the conjunctiva of the eye and subcutaneous tissue were identified as *D. repens* on the basis of the morphological keys by Miyazaki in 1991 [11]. Molecular characterization was conducted as follows: genomic DNA was extracted from individual parasites using a Qiagen genomic DNA extraction Kit (Qiagen, Valencia, California, USA). Extracted genomic DNA was diluted to a working concentration of 100 ng/μl, and 1 μl of this was used in 50 μl PCR reaction volume. PCR amplified a fragment of the cytochrome *c* oxidase subunit 1 (*cox1*), using the UCO1F1-UCO1R2 primer pairs and additionally as previously described as follows [9,12]: UCO1F1:

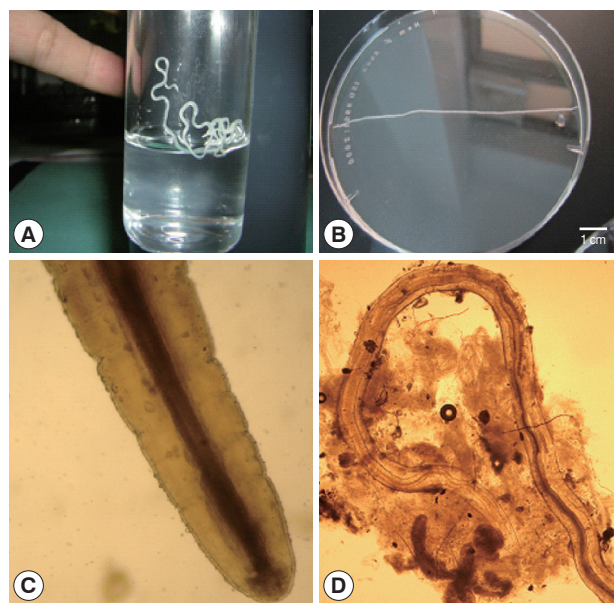


Fig. 1. *Dirofilaria repens* collected from the conjunctiva (A, B) and subcutaneous tissue (C, D) of humans in Vietnam. (A) A female worm from the conjunctiva of a patient. (B) Another specimen from another patient. (C) Anterior end of a worm showing the mouth and esophagus. × 100. (D) Posterior part of a female worm extracted from the subcutaneous tissue of a patient. × 40.

Table 2. Sequencing of the portion *cox1* of different filarial species from GenBank compared with *Dirofilaria repens** in Vietnam

Notation	Origin	Host	Length	Species	GenBank	Author
GCA-VN1	Vietnam	Human	461 bp	<i>Dirofilaria repens</i> *	-	De, Hoa, and Chai ^a
GCH-VN2	Vietnam	Human	461 bp	<i>Dirofilaria repens</i> *	-	De, Hoa, and Chai ^a
GCD-VN3	Vietnam	Human	461 bp	<i>Dirofilaria repens</i> *	-	De, Hoa, and Chai ^a
Drep (ITA1)	Italy	-	461 bp	<i>Dirofilaria repens</i>	AJ271614	[8]
Drep (ITA2)	Italy	-	461 bp	<i>Dirofilaria repens</i>	DQ358814	Serini et al. (GenBank)
Dimm	Australia	Dog	461 bp	<i>Dirofilaria immitis</i>	AJ537512	[14]
Bmal	GenBank	-	461 bp	<i>Brugia malayi</i>	AF538716	[15]
Ovol	GenBank	-	461 bp	<i>Onchocerca volvulus</i>	AF015193	[16]

^aResults of the present study.

A Nucleotide

		*	120	*	140	*	160	*	180	*	200	
GCA-VN1	:	AGAATATACGTTCTACTGCTGTTACTTTAGATCAAATTTAGTATGTTTGTGTTGAACCTCTTATTAACTCTTTTCTTTAGTTTATCTGTTCTCTGTTTT	:	200								
GCH-VN2	:	:	200								
GCD-VN3	:	:	200								
Drep (ITA1)	:G.....G.....G.....	:	200								
Drep (ITA2)	:G.....GG.....G.....	:	200								
Dimm	:A.....G.....T.A..G..A..G..A..G.....	:	200								
Bmal	:	..A...G...GT...C...GGCA...A...G...G...T.GC.G..G...A.....	:	200								
Ovo1	:G...G...G.....G...G...T.A...G.....	:	200								

		*	220	*	240	*	260	*	280	*	300	
GCA-VN1	:	AGCTGGTTCTTTGTGTTTTATTGTTGGATCGTAATTTTAACTTCTTTTTATGATACTAAAAGGTTGGTAATCCTTTATTGTATCAGCATTATT	:	300								
GCH-VN2	:	:	300								
GCD-VN3	:	:	300								
Drep (ITA1)	:	G.....G..A..A.....G.....G.....G.....	:	300								
Drep (ITA2)	:G..A..A.....G.....G.....G.....	:	300								
Dimm	:	G.....A..A.....G.....G...T...G.....G.....	:	300								
Bmal	:	G..A.....C...T.....T.....A.....	:	300								
Ovo1	:	G.....A...G.....G...G.....G.....G.....	:	300								

		*	320	*	340	*	360	*	380	*	400	
GCA-VN1	:	TGATTTTTTGGTCACTCTGAGGTTTATGTTATTATTTTACCTGTTTTTGGTATTATTAGTGAATGTGTTTGTTTTTAACTGATAAGGATCGTTTGGTTG	:	400								
GCH-VN2	:	:	400								
GCD-VN3	:	:	400								
Drep (ITA1)	:G.....G.....	:	400								
Drep (ITA2)	:G.....G.....	:	400								
Dimm	:A.....G...G...G...G.....A...G.....	:	400								
Bmal	:	..G.....G.C.A.A.....C.....GGC.....C.....C.....A.....	:	400								
Ovo1	:G.....G.GGC.....A...G.....	:	400								

		*	420	*	440	*	460	
GCA-VN1	:	GTCAGACTAGAA TAACCTTTGCCTCTATTGGATTGCTGTTTTAGTACTTCTGTTTGGGC	:	461				
GCH-VN2	:	:	461				
GCD-VN3	:	:	461				
Drep (ITA1)	:T.....T.....A.G	:	461				
Drep (ITA2)	:T.....T.....A.G	:	461				
Dimm	:	..C.....T..G.....T.....A..G..G.....G	:	461				
Bmal	:A.....T..G.....T.....A.....C.....A.....A.G	:	461				
Ovo1	:G..G.....T.....A.....G	:	461				

B Amino acid

		*	20	*	40	*	60	*	80	*	
GCA-VN1	:	DGQPELSLD SMILGLHTVGI G SLLGAINFMVTTQNMRSTAVTLDQI SMFVWTSYLT S FLLVLSV FVLG S LLLFL LDRNFNT SFYDTKKG	:	90							
GCH-VN2	:	:	90							
GCD-VN3	:	:	90							
Drep (ITA1)	:	:	90							
Drep (ITA2)	:	:	90							
Dimm	:	E.....V.....AN..	:	90							
Bmal	:	E.....V.....F.....V.....A.....F.....N..	:	90							
Ovo1	:	E.....	:	90							

		*	100	*	120	*	140	*
GCA-VN1	:	GNPLLYQHLEWFFGHPEVYVI ILPVFGI ISECVLFLTDKDRLFCGQTSMTFAS IWI IAVLGT SVW	:	153				
GCH-VN2	:	:	153				
GCD-VN3	:	:	153				
Drep (ITA1)	:	:	153				
Drep (ITA2)	:	:	153				
Dimm	:	:	153				
Bmal	:A.....	:	153				
Ovo1	:A.....	:	153				

Fig. 2. Comparison of 461 nucleotide (A) and 153 amino acids (B) of portion *cox1* mitochondrial genome between Vietnamese *Dirofilaria* and other species of the family Filaridae, including the Italian *Dirofilaria repens* (Drep [ITA1] and Drep [ITA2]), Australian *D. immitis* (Dimm), *Brugia malayi* (Bmal), and *Onchocerca volvulus* (Ovo1). Note differences between the Vietnamese *Dirofilaria* (GCA-VN1; GCH-VN2; GCD-VN3) and other species showed by sign nucleotide (or amino acid) of them; mark (.) is similar each other in nucleotide (or amino acid).

5'GGTGTGGTTGAACCTTTTATCCTCC3' and UCO1R2: 5'CC-AACCATAAACATATGATGAGCCCA3'.

PCR products purified using a QIAquick Purification Kit (Qiagen) were subjected to direct sequencing using the Big-Dye Terminator Cycle Sequencing technology on an automated sequencer, ABI 3100 Avant Genetic Analyzer (Applied Bio-

systems, Foster City, California, USA). Sequences were then edited using SeqEdv1.03, aligned using Assembly LIGNv1.9c, and analyzed using the MacVector 8.2 package (Accelrys Inc., San Diego, California, USA). Sequences were searched against the GenBank database, using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>), and approximately 500

Table 3. Percentage identity of nucleotide (above diagonal) and amino acid homology (below diagonal) of *cox1* sequences of Vietnamese *Dirofilaria repens* and other Filaridae in GenBank

	GCA-VN1	GCH-VN2	GCD-VN3	Drep (ITA1)	Drep (ITA2)	Dimm	Bmal	Ovol
GCA-VN1		100	100	95	95	89	87	91
GCH-VN2	100		100	95	95	89	87	91
GCD-VN3	100	100		95	95	89	87	91
Drep (ITA1)	100	100	100		99	90	87	92
Drep (ITA2)	99	99	99	99		90	87	92
Dimm	96	96	96	96	96		83	89
Bmal	94	94	94	94	94	92		86
Ovol	98	98	98	98	97	96	95	

GCA-VN1, GCH-VN2, and GCD-VN3 were Vietnamese *Dirofilaria*; Drep (ITA1) and Drep (ITA2) were Italian *Dirofilaria repens* (GenBank no. AJ271614 and DQ358814); Dimm: *Dirofilaria immitis* (GenBank no. AJ537512); Bmal: *Brugia malayi* (GenBank no. AF538716); Ovol: *Onchocerca volvulus* (GenBank no. AF015193).

bp of the *cox1* of *D. repens* from Italy and others sequences were used for comparative purposes. The *cox1* sequences of the Vietnamese *Dirofilaria*, including parasites from the conjunctiva (GCA-VN1 and GCH-VN2) and subcutaneous tissue (GCD-VN3), were compared with *D. repens* from Italy (ITA1 and ITA2), *D. immitis* from Australia (Dimm), *Brugia malayi* (Bmal), and *Onchocerca volvulus* (Ovol), using GENEDOC2.5 and MEGA3.1 (Table 2).

PCR products (500 bp of *cox1*) were successfully sequenced, using UCO1F1 and UCO1R2 primers. A portion of *cox1* from the Vietnamese *Dirofilaria cox1*, including 461 nucleotides (A) and 153 amino acids (B), were compared with the *cox1* from Italian *D. repens* (Drep [ITA1] and Drep [ITA2], Australian *D. immitis* [Dimm], *B. malayi* [Bmal], and *O. volvulus* [Ovol]) (Table 3; Fig. 2).

The 3 portions of the *cox1* sequences of the Vietnamese *Dirofilaria* exhibited a 95% nucleotide and 99-100% amino acid identity with the Italian *D. repens* (GenBank no. AJ271614 and DQ358814). In contrast, these sequences exhibited a 89% nucleotide and 96% amino acid identity with the Australian *D. immitis*; 87% (nucleotide) and 94% (amino acid) similarity with *B. malayi* (AF538716); and 91% (nucleotide) and 98% (amino acid) similarity with *O. volvulus* (AF015193) (Table 3; Fig. 2). The phylogenetic analysis results are shown in Fig. 3. Consequently, we characterized the species of filaria samples from the eye and subcutaneous tissue of patients in Vietnam as *D. repens* (Nematoda: Filarioidea).

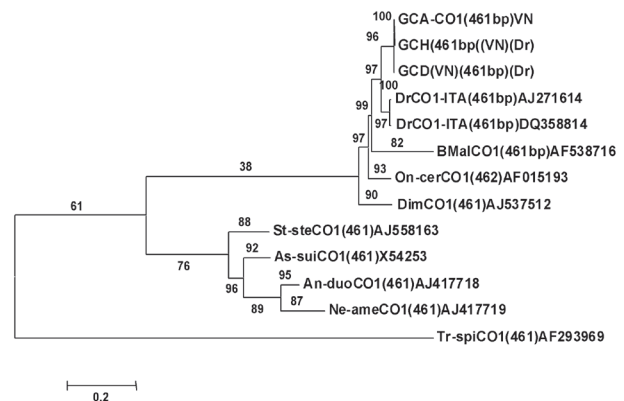


Fig. 3. The phylogenetic tree based on a portion of *cox1* sequence of *Dirofilaria* isolates and nematode strains, including 3 collected in Vietnam. Topology was constructed by MEGA 4.1 using the neighbor-joining method [17]. *Dirofilaria repens* from this study and 2 from Italy are shown by the vertical bar. The length of the *cox1* sequence is indicated in brackets. Bootstrap values (%) are indicated in numerals from 1,000 replicates. GCA-VN1, GCH-VN2, and GCD-VN3=Vietnamese *Dirofilaria*; Drep (ITA1) and Drep (ITA2)=Italian *Dirofilaria repens* (GenBank no. AJ271614 and DQ358814); Dimm: *Dirofilaria immitis* (GenBank no. AJ537512); Bmal: *Brugia malayi* (GenBank no. AF538716); Ovol: *Onchocerca volvulus* (GenBank no. AF015193); St-ste=*Strongyloides stercoralis* (GenBank no. AJ558163); As-sui=*Ascaris sum* (GenBank no. X54253); An-duo=*Ancylostoma duodenale* (GenBank no. AJ417718); Ne-ame=*Neocator americanus* (GenBank no. AJ417719); Tr-spi=*Trichinella spiralis* (GenBank no. AF293969).

DISCUSSION

In this study, 10 *Dirofilaria* worm specimens from humans, including 9 from the conjunctiva and 1 from the subcutaneous tissue, were identified by the morphology and molecular methods as *D. repens*. In Vietnam, the filarial worm of this species was first reported from a human conjunctiva in 2008 [12], and another was reported from the human subcutaneous tissue in 2010 [13]. This is the 3rd report of human *D. repens* infection in Vietnam which involved the conjunctiva or subcutaneous tissue.

This species is parasitic in dogs, cats, and wild animals [1], and together with *D. immitis* it can cause complicated epidemiology in zoonotic diseases. Dirofilariasis is transmitted to humans by mosquitoes, including *Anopheles*, *Culex*, and *Aedes* [2], and these mosquitoes are common in Vietnam. Feeding dogs and cats are very common in the whole country. Thus, a high risk for human dirofiliasis is existing everywhere in Vietnam.

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