Genetic diversity of *Dirofilaria* spp. isolated from subcutaneous and ocular lesions of human patients in Ukraine.

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Abstract

This short communication describes the phylogenetic analysis of 48 *Dirofilaria* worms isolated from human patients in Ukraine. 102 cases were both of subcutaneous (47; 46.1%) and ocular (54; 52.9%) locations. Worms from 44 patients (15 subcutaneous and 29 ocular) were subjected to DNA extraction and amplification of a specific fragment of the 12S rRNA subunit, and sequences were used for phylogenetic analysis. Results showed that 13.8% of the ocular cases analyzed at molecular level were caused by *D. immitis*. Very few cases of ocular human dirofilariosis due to *D. immitis* have been described in the literature to date, majority of them attributed to *D. repens*. Our results show that ocular dirofilariosis cannot be excluded in areas of low endemicity for *D. repens* where *D. immitis* is also present.

Keywords: *Dirofilaria repens*; *Dirofilaria immitis*; Ukraine; 12S RNA; Subcutaneous; Ocular.

Footnote: Sequences described in this work have been deposited in GenBank under accession numbers KM205372 to KM205415.

1. Introduction

Dirofilariases are vector borne transmitted zoonoses caused by different species of the genus *Dirofilaria* of which the main reservoirs are domestic and wild canids. Cases of human dirofilariosis are described in areas where infected animals are found and where the culicid mosquitoes feed on animals and humans. *Dirofilaria immitis* is the causal agent of human pulmonary dirofilariosis worldwide. Human subcutaneous and ocular dirofilariosis is usually caused by *Dirofilaria repens* in the Old World and by *Dirofilaria tenuis*, *Dirofilaria conjunctivae* and *Dirofilaria ursi* in North America. Both *D. immitis* and *D. repens* have been reported in anatomic sites other than the above-mentioned far less often (Simón *et al.*, 2012).

Human subcutaneous and ocular dirofilariosis caused by *D. repens* has been detected with increasing frequency in the Eurasian continent in the last few years, and it has been categorized as emerging disease in Europe (Genchi *et al.*, 2011a). This spreading is attributed to the global warming and the lack of an adequate treatment of pets in some countries (Kartashev *et al.*, 2014). Around 4000 human cases have been reported worldwide, 3500 of which have been diagnosed in Europe, most of them in Ukraine and the Russian Federation during the last 10 years (Kartashev *et al.*, 2014; Salamatina *et al.*, 2013). Ocular cases are the most frequently reported, making up 35% of the human cases described worldwide (Genchi *et al.*, 2011b) and more than 40% of the cases found in Ukraine and the Russian Federation (Avdiukhina *et al.*, 1996; Iliasov *et al.*, 2013). Conversely, only 380 cases of human pulmonary dirofilariosis caused by *D. immitis* have been reported in the literature, most of them in the Americas and Japan, 33 of which have been diagnosed in European countries (Simón *et al.*, 2012).

In the present work, 48 worms surgically removed from patients resident in Ukraine with subcutaneous nodules or ocular affection were characterized by molecular techniques. These techniques allowed us to identify an unusually high prevalence of ocular cases caused by *D. immitis* not reported previously in any other endemic area.

2. Material and Methods

A total of 102 human patients were diagnosed of dirofilariosis in several hospitals from different territories of Ukraine in 2010. *Dirofilaria* spp. worms were isolated either from subcutaneous locations (47 samples; 46.1% of cases), from ocular and periocular

1 locations (conjunctiva or eyelids; 54 samples; 52.9% of cases), or from both locations in
2 the same patient (1 case). Patients were subjected to X-ray examination and none of them
3 showed lung lesions. Worms collected from 44 patients in subcutaneous (15 samples) or
4 ocular and periocular locations (conjunctiva or eyelids; 29 samples) were preserved in
5 ethanol. Preserved parasites were washed twice with cold PBS and subjected to DNA
6 extraction with the Nucleospin® Tissue Kit (Macherey-Nagel), following the
7 **manufacturer's instructions**. Extracted DNA was amplified by PCR using primers specific
8 for the 12S rDNA region, similar to those described and used for *Dirofilaria* species
9 identification by Casiraghi *et al.* (2004) and Gioia *et al.* (2010), annealing in a stretch of
10 the 12S sequence that has shown to be highly conserved in nematodes. Primers were
11 designed after alignment of the 12S rDNA partial sequences of *D. immitis* (GenBank
12 accession number EU169125) and *D. repens* (GenBank accession number GQ292761), in
13 regions conserved in both species. Alignment was done with the Multalin facility at
14 <http://multalin.toulouse.inra.fr/multalin/multalin.html>. The designed primers were 12SF
15 (5'-GTTCCAGAATAATCGGCTATAC) and 12SR (5'-
16 ATTGACGGATGGTTTGTACC), comprising a sequence of 514 base pairs (bp) for *D.*
17 *immitis* and of 509 bp for *D. repens*. The PCR reaction was performed in a final volume of
18 25 µl containing 2.5 µl of 10x PCR buffer with 15 mM MgCl₂, 2 µl of MgCl₂ 25 mM, 0.5
19 µl dNTPs 10 mM, 25 nM of each primer, 0.1 µl High Fidelity DNA polymerase
20 (Fermentas), 18.7 µl sterile distilled water and 1 µl of purified DNA. PCR conditions were
21 as follows: 30 cycles of 95°C for 30 seg, 46°C for 30 seg and 72°C for 1 min, with a final
22 extension step of 72°C for 5 min.

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41 PCR products were loaded in 1% agarose gels with ethidium bromhyde and bands
42 at the expected molecular weight were excised from the gel. Bands were purified with the
43 Gene Jet Gel Extraction Kit (Thermo Scientific), following the manufacturers' **instructions**.
44 Purified bands were cloned in the pSC-A vector with the Strataclone PCR cloning kit
45 (Agilent Technologies) as indicated by the manufacturers. Ligation reactions were used to
46 transform *Escherichia coli* XL1-B cells. Cells were grown in LB-agar plates with 50 µg/ml
47 ampicillin overnight at 37°C. Five colonies for each PCR product were grown overnight in
48 liquid LB plus ampicillin at 37°C with shacking. Cells were pelleted by centrifugation and
49 the plasmids were extracted with the Nucleospin Plasmid kit (Machery-Nagel). Extracted
50 plasmids were sequenced with the universal primers T3 and T7.

Obtained sequences were subjected to similarity analysis with sequences deposited in GenBank (nrNCBI) using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic relationships among our isolates and those *Dirofilaria* isolates described before, were evaluated comparing the obtained sequences with the following 12S rRNA sequences available in GenBank: AM779778 (*D. repens*, subcutaneous human isolate, Italy), AM779777 (*D. repens*, cat isolate, Italy), KC953031 (*D. repens*, dog isolate, Turkey), AM779776 (*D. repens*, dog isolate, Italy), AM779773 (*D. repens*, cat isolate, Italy); AM779774 (*D. repens*, subcutaneous human isolate, Italy), GQ292761 (*D. repens*, subcutaneous human isolate, India), AM779775 (*D. repens*, dog isolate, Italy), HQ540423 (*D. immitis*, ocular human isolate, Brazil), EU169125 (*D. immitis* dog isolate, China), AM779771 (*D. immitis*, cat isolate, Italy), EU182328 and EU182327 (*D. immitis*, red panda isolates, China), AM779770 and FN391554 (*D. immitis*, dog isolates, Italy), AM779769 (*D. immitis*, cat isolate, Italy), and JN801161 (*Ascaris lumbricoides*; outgroup). For phylogenetic analysis sequences were aligned using the Clustal_X software (Thompson *et al.*, 1997). The distances were calculated according to Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees of 12S rRNA gene were inferred using the neighbour-joining analysis (Saitou & Nei, 1987), and maximum likelihood (Rogers & Swofford, 1998). MEGA5 software (Tamura *et al.*, 2011) was used for all analyses.

subcutaneous worms was higher for worms displaying the sequence of 509 bp (6 out of 8; 75%) than for worms with the sequence of 510 pb (19 out of 32; 59%), as shown in Figure 1. Whether those genetic differences influence the anatomical location of *D. repens* worms should be further analyzed with an extended number of isolates.

The remaining four sequences (isolates 23, 25, 48 and 84) showed identities ranging from 100 to 95% with 12S rRNA sequences of *D. immitis* isolates, and $\leq 91\%$ identity with the same type of sequence from *D. repens*, and thus they were classified as *D. immitis*. The four isolates corresponded with worms found in ocular locations, and showed identical 514 bp in length sequences among them, apart from bases at positions 241 and 437 that were different in isolate number 84 when compared to isolates 23, 25 and 48.

Phylogenetic analysis showed the analyzed sequences separated in two main branches, corresponding to the two abovementioned species (Figure 1). The neighbour-joining analysis and maximum likelihood analysis resulted in trees with similar topology (data not shown).

D. repens and *D. immitis* are maintained in the same animal reservoirs in the Old World. The epidemiological situation in animals was assessed in the Rostov region of Russia close to Ukraine, showing that around 20% of dogs tested positive in the Knott test for *Dirofilaria* (Kartashev *et al.*, 2011). Species identification in the infected dogs showed that *D. repens* and *D. immitis* had prevalence rates of 44.7% and 30.3%, respectively, and the remaining dogs had mixed infections (Kartashev *et al.*, 2011). Nevertheless, reported cases of human infection due to *D. repens* widely predominate even in areas with high endemicity of *D. immitis* in animal reservoirs (rev. in Simon *et al.*, 2012).

Epidemiological data on human dirofilariosis in Ukraine and Southwestern Russia also confirm the predominance of *D. repens* over *D. immitis* in human infections. In this area, the vast majority of registered cases of human dirofilariosis were subcutaneous or ocular (attributed to *D. repens*), with only 2 cases of human pulmonary dirofilariosis (attributed to *D. immitis*) reported until now (Iliasov *et al.*, 2013; Kartashev *et al.*, 2011; Salamatin *et al.*, 2013; Simón *et al.*, 2012). Here, we only analyzed subcutaneous and ocular affected patients. The proportion of patients was slightly higher for the ocular location in accordance with previous analysis in the region (Avdiukhina *et al.*, 1996; Iliasov *et al.*, 2013; Salamatin *et al.*, 2013).

Ocular dirofilariosis caused by *D. immitis* has been reported only occasionally, both in animal and human infections. In the New World, ocular infection with *D. immitis* in the animal reservoir (dog) has been more frequently reported than in the Old World (e.g., Eberhard *et al.*, 1977; Guterbock *et al.*, 1981; Lavers *et al.*, 1969; Mallet *et al.*, 1971). In Europe, the first documented case of ocular *D. immitis* in a dog was reported in 2009 in the south-east of Italy (Dantas-Torres *et al.*, 2009). In human patients, very few cases of ocular *D. immitis* have been reported both in the New World (Moorhouse, 1978 in Australia; Otranto *et al.*, 2011 in northern Brazil) and in the Old World (Avellis *et al.*, 2011 in northeast Italy). Thus, the most striking finding during our analysis was the identification of *D. immitis* in four of the 44 isolates –9% over the total–, all them found in ocular locations and thus accounting for the 13.8% of the ocular isolates analyzed here.

Only two of the above-mentioned parasite isolated from ocular cases due to *D. immitis* were subjected to 12S rRNA specific PCR and sequencing: the Italian isolate from a dog (Dantas-Torres *et al.*, 2009; GenBank accession number FN391554) and the Brazilian isolate from a human patient (Otranto *et al.*, 2011; GenBank accession number HQ540423). The BLAST identities and the phylogenetic analysis done here, show that those two previously analyzed isolates group with *D. immitis* sequences (Fig. 1). Nevertheless, the sequence derived from the human ocular isolate of Brazilian origin was slightly different than the rest of *D. immitis* sequences. Similarly, Otranto *et al.*, (2011) pointed out that this human isolate was distinct from the *D. immitis* reference sequences, including those of *D. immitis* infesting dogs in the same area, and concluded that this isolate could be a different zoonotic *Dirofilaria* species, e.g., an as yet unknown species infesting wild mammals in Brazil (Otranto *et al.*, 2011). Similarly, two isolates of *D. repens* (GQ292761 and AM779775) lied apart from the rest of *D. repens* isolates used for the phylogenetic analysis (Fig. 1). The resolution power of the 12S rRNA fragment

analyzed here at species level for nematodes, together with the identity close to 100% of the majority of isolates in each species, could indicate that those divergent isolates described before represent other genetic lineages, but no 12S sequences from species of the genus *Dirofilaria* different from *D. immitis* or *D. repens* are currently available. A common genetic marker for nematodes should be investigated in the available isolates of *Dirofilaria* spp. to better characterize the zoonotic potential of the different *Dirofilaria* species maintained in domestic and wild animal reservoirs.

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Legends for figures

Figure 1. Maximum likelihood phylogenetic rooted tree based on 12S rDNA gene sequences showing the taxonomic affiliation of the isolates. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. For each isolate, Genbank Accession number, isolate number (for Ukrainian isolates), species, host, country and anatomical location are detailed.

Footnote for Figure 1: HU, human; UA, Ukraine; IT, Italy; TR, Turkey; IN, India; BZ, Brazil; CH, China; SC, subcutaneous; OC, ocular.

Figure
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