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Short Communication

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 *Dirofilaria 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 *Dirofilaria repens*. Our results show that ocular dirofilariosis cannot be excluded in areas of low endemicity for *D. repens* were *D. immitis* is also present.

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; Sałamatin 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. Materials and methods

A total of 102 human patients were diagnosed of dirofilariosis in several hospitals from different territories of Ukraine in 2010.

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

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Dirofilaria spp. worms were isolated either from subcutaneous locations (47 samples; 46.1% of cases), from ocular and periocular locations (conjunctiva or eyelids; 54 samples; 52.9% of cases), or from both locations in the same patient (1 case). Patients were subjected to X-ray examination and none of them showed lung lesions. Worms collected from 44 patients in subcutaneous (15 samples) or ocular and periocular locations (conjunctiva or eyelids; 29 samples) were preserved in ethanol. Preserved parasites were washed twice with cold PBS and subjected to DNA extraction with the Nucleospin® Tissue Kit (Macherey-Nagel), following the manufaturer's instructions. Extracted DNA was amplified by PCR using primers specific for the 12S rDNA region, similar to those described and used for *Dirofilaria* species identification by Casiraghi et al. (2004) and Gioia et al. (2010), annealing in a stretch of the 12S sequence that has shown to be highly conserved in nematodes. Primers were designed after alignment of the 12S rDNA partial sequences of *D. immitis* (GenBank accession number EU169125) and *D. repens* (GenBank accession number GQ292761), in regions conserved in both species. Alignment was done with the Multalin facility at http://multalin.toulouse.inra.fr/multalin/multalin.html. The designed primers were 12SF (5′-GTTCCAGAATAATCGGCTATAC) and 12SR (5′-ATTGACGGATGGTTTGTACC), comprising a sequence of 514 base pairs (bp) for *D. immitis* and of 509 bp for *D. repens*. The PCR reaction was performed in a final volume of 25 μl containing 2.5 μl of 10× PCR buffer with 15 mM $MgCl_2$, 2 μl of $MgCl_2$ 25 mM, 0.5 μl dNTPs 10 mM, 25 nM of each primer, 0.1 μl High Fidelity DNA polymerase (Fermentas), 18.7 μl sterile distilled water and 1 μl of purified DNA. PCR conditions were as follows: 30 cycles of 95 °C for 30 s, 46 °C for 30 s and 72 °C for 1 min, with a final extension step of 72 °C for 5 min.

PCR products were loaded in 1% agarose gels with ethidium bromide and bands at the expected molecular weight were excised from the gel. Bands were purified with the Gene Jet Gel Extraction Kit (Thermo Scientific), following the manufacturers' instructions. Purified bands were cloned in the pSC-A vector with the Strataclone PCR cloning kit (Agilent Technologies) as indicated by the manufacturers. Ligation reactions were used to transform *Escherichia coli* XL1-B cells. Cells were grown in LB-agar plates with 50 μg/ml ampicillin overnight at 37 °C. Five colonies for each PCR product were grown overnight in liquid LB plus ampicillin at 37 °C with shacking. Cells were pelleted by centrifugation and the plasmids were extracted with the Nucleospin Plasmid kit (Macherey-Nagel). Extracted 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 and Nei, 1987), and maximum likelihood (Rogers and Swofford, 1998). MEGA5 software (Tamura et al., 2011) was used for all analyses.

3. Results and discussion

A fragment of the 12S rRNA subunit was amplified out of 44 *Dirofilaria* worms isolated from subcutaneous ($n = 15$; 34%) and ocular ($n = 29$; 66%) locations in Ukrainian patients. Corresponding sequences were compared with those deposited in GenBank, allowing the identification of each isolate at species level. 40 of the isolates (91%) with ocular ($n = 25$) and subcutaneous ($n = 15$) locations showed 100–97% identities in BLAST with the first eight matches, corresponding to 12S rRNA sequences of *D. repens* isolates and 91% identity with the ninth match belonging to *D. immitis*, and thus were classified as *D. repens*. This group of 40 sequences only differed in one base: 8 sequences were 509 bp in length (isolates 15, 21, 35, 50, 56, 74, 77 and 82) and 32 sequences 510 bp in length showing one additional base at position 88. The proportion of ocular worms vs. 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 bp (19 out of 32; 59%), as shown in Fig. 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 ≤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 above-mentioned species (Fig. 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 Simón 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; Sałamatin 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; Sałamatin 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

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