Human Dirofilariosis diagnosis by specific DNA marker identification

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SUMMARY

Pulmonary dirofilariosis is a rare benign condition. Transmitted by mosquitoes to humans that results in peripheral pulmonary nodules, it is caused by the dog worm Dirofilaria Railliet et Henry, 1911 (Nematoda Onchocercidae). Dirofilaria repens Railliet et Henry, 1911 in humans is usually characterized by the presence, after the penetration of the larva L3, of a single subcutaneous nodule near the point of the bite of the vector mosquito. In the rare cases of pulmonary infection, the nematode is usually in an advanced state of decomposition in a thrombosed arteriole, forming a small roundish infarct area. Generally, it is presumed to be neoplastic, requiring biopsy or surgery before being diagnosed correctly. A specific molecular test was developed for the detection of specific DNA targets for differential diagnosis between Dirofilaria repens and D. immitis.

KEY WORDS
Pulmonary dirofilariosis; nematodes; DNA extraction.

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INTRODUCTION

Human dirofilariosis is a zoonotic infection caused by Onchocercidae Nematodes Dirofilaria repens Railliet et Henry, 1911 and D. immitis Leidy, 1856. During a blood meal, an infected mosquito (Aedes, Culex, Anopheles, Mansonia) introduces third-stage filarial larvae of Dirofilaria Railliet et Henry, 1911 into the skin of the definitive host, which are usually domestic and wild carnivores living in tropical and temperate regions, where they penetrate into the bite wound. In the definitive host, larvae develop to mature worms producing microfilariae that may be found in peripheral blood. The main natural hosts for the Dirofilaria species that most frequently cause disease in humans are canids. Humans can be accidentally infected with Dirofilaria larvae through mosquito bites. The infection can result in subcutaneous or conjunctival nodules. Besides this, a more typical localization of the Dirofilaria species have been shown to infect viscera (lungs and mesentery), female breast, and male genitalia.

As for the D. repens infection, it is usually characterized by the occurrence, 2 to 12 months after the L3 penetration, of a usually single nodule near the point of the mosquito vector bite, which can be accompanied by urticarial manifestations. More deeply located nodules are often misidentified as malignant tumours, requiring biopsy or surgery before being correctly diagnosed.

We report here a rare human case, observed in an Italian woman, of double pulmonary nodules mimicking neoplasm, in which the molecular identification of D. repens was performed on surgical specimen through mini invasive intervention.
odrop ND-J000 through the absorbance reading at 260 nm and employed in PCR test targeted to the spacer sequences included in the ribosomal regions. Reference genomic DNA was extracted from animal blood containing *D. repens* (15,000 mff/ml), using the commercial DNeasy Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany).

We used the cytochrome C oxidase subunit 1 (cox1) as species-specific marker to obtain different sizes amplicones: 169 bp for *Dirofilaria repens*, 479 bp for *Dirofilaria repens*, 589 bp for *Acanthochelionema reconditum* (Grassi, 1890), and 304 bp for *Cercopithifilaria* sp. respectively. The mitochondrial gene for cytochrome c oxidase subunit 1 (cox1) can be amplified in a single-step multiplex PCR using a set of species-specific forward primers paired with a single reverse primer in accord to Shibasaki et al. (1997). The estimation of molecular distance based on the DNA sequencing indicated that this gene is useful as a marker for the barcoding of these nematodes to species level.

A quantity of 20 ng DNA was used for the amplification with four specific forward primers and a common reverse primer as in Table 1.

The mixture was optimized as follows: 1X PCR Buffer II (Thermo), 2mM MgCl₂, 1mM dNTP mixture, 0.4 μM Arcox1F, 0.4 μM Cbcx1F, 0.4 μM Dicox1F, 0.4 μM Drcox1F, 1.2 μM NTR rev primer, 2U taq Polymerase (Thermo), and water for a 50 μL final volume. The amplification was optimized in accord to the manufacturer (Thermo), in a 9700 thermal cycler (Applied Biosystems). An initial denaturation step (94 °C for 8 min) was followed by 35 cycles (with each cycle consisting of DNA denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min) and a final extension step at 72 °C for 7 min. A no-template negative control was included in each PCR run. The PCR products were purified with a Spin PCR purification kit (QIAGEN), following the manufacturer’s protocol. The DNA was eluted in a 25 μl elution buffer and sequenced with the species-specific primers using BigDye Terminator cycle sequencing 1.1 kit (Thermo). The capillary electrophoresis was performed on an ABI Prism 3130 DNA sequencer (Life technologies). The obtained data were analysed by the Wu Blast 2 sequence alignment software. We considered a homology of 97% as stringent parameter for strain identification.

The bibliography consulted for this work, in addition to the one mentioned, is listed in the references.

**RESULTS**

Multiplex-PCR cox1 amplicons from DNA extracted from the embedded tissue resulted in a band of expected size for *D. repens* (479 bp). Neither non-specific bands nor amplification products from negative control samples were detected. The BLAST

![Figure 1](image.png)

Figure 1. Agarose gel electrophoresis. Lane 1, lane 2, lane 3, lane 4, lane 5, PCR products; Lane 6, + control; Lane 7, - ve control; Lane L, 100 bp molecular weight marker.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>Arc ox1F</td>
<td>5’-ATC TTT GGT TAT GGT GTA TC-3’</td>
</tr>
<tr>
<td>Cbc ox1F</td>
<td>5’-CGG GTC TTT TTG ATT ATT GC-3’</td>
</tr>
<tr>
<td>Dicox1F</td>
<td>5’-ACC GGT GTG TGG GAT TGG TA-3’</td>
</tr>
<tr>
<td>Drcox1F</td>
<td>5’-GTA TA TTT TGG GTT TAC ATA CTG TA-3’</td>
</tr>
<tr>
<td>Common reverse primer NTR</td>
<td>5’-ATA AGT ACG AGT ATC AAT ATC-3’</td>
</tr>
</tbody>
</table>

Table 1. Species-specific primer sets to amplify cox1 gene regions of different sizes.
human dirofilariosis diagnosis by specific DNA marker identification to be possible causes of the spread of vector-borne diseases. Human dirofilariosis is currently considered an emergent zoonosis in Italy, France, Hungary, and Russia.

Woman appear to be more commonly affected than men, although there is no statistical difference. Besides subcutaneous and ocular localization, *Dirofilaria* sp. have been shown to infect viscera (lung and mesentery), female breast, and male genitalia. About 27 cases of pulmonary localization have been reported from 1981 to 2010. Many benign forms of infection go unnoticed due to a lack of awareness among the medical profession. Cases of deep tissues localization usually evoke concern that lead to a clinical suspicion of neoplasia requiring biopsy or more invasive surgery for histological assessment as only possibility for differential diagnosis (until the recent introduction of molecular methods based on polymerase chain reaction and sequencing). Our case of human pulmonary dirofilariosis was diagnosed using molecular diagnostic test. In order to

### DISCUSSION

Growing concern over *D. repens* into endemic areas of Southern Europe has been confirmed by recent increasing numbers of published human cases in Southern Europe, with particular rising prevalence in Italy (4.5 per year from 1986–1998 to 15.6 per year from 1999–2009). Furthermore, the spread of the infection from the Southern Mediterranean regions toward the Northern and Eastern areas has been observed. Climate changes, insecticide resistance, and improvement of transport networks are thought to be possible causes of the spread of vector-borne diseases. Human dirofilariosis is currently considered an emergent zoonosis in Italy, France, Hungary, and Russia.

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provide a definitive diagnosis on the molecular level, a range of PCR assays has been developed to detect the filarial DNA in definitive hosts.

CONCLUSIONS

Even though dirofilariasis is still a rare condition, its increasing incidence must lead us to include it in differential diagnosis of subcutaneous and visceral nodules of unknown origin, especially in endemic areas. We encourage minimally invasive techniques to perform therapeutic and diagnostic excision of the nodules. We emphasize the importance of biomolecular assays to achieve etiological diagnosis.

REFERENCES