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Human Dirofilariosis diagnosis by specific DNA marker identification

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Summary

Pulmonary dirofilariasis is a rare benign condition. Transmitted by mosquitos 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 dirofilariasis; 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.

Material and Methods

The biological sample was a paraffin embedded tissue taken from a subcutaneous nodule of a patient. Total DNA extraction was carried out by using a QIAmp DNA mini kit (QIAGEN). The contaminations were excluded during the DNA extraction, amplification or elution steps by application of the good laboratory practice. The DNA concentration was evaluated by the use of the spectrophotometer Nan-

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 *Acanthocheilonema 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 , 1mMd NTP mixture, 0.4 µM Arcox1F, 0.4 µM Cbcox1F, 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 analised by the Wu Blast 2 sequence allignment 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

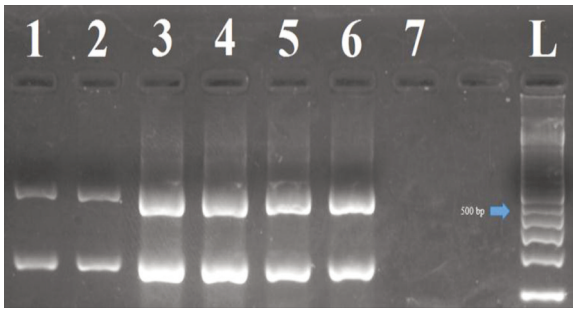

Figure 1. Agarose gel electrophoresis. Lane 1, lane 2, lane 3, lane 4, lane 5, PCR products; Lane 6, + control; Lane 7, - ve control; Lane L, 100 bp molecular weight marker.

Primers name	*Primer sequences*
Arcox1F	5'-ATC TTT GTT TAT GGT GTA TC-3'
Cbcox1F	5'-CGG GTC TTT GTT GTT TTT ATT GC-3'
Dicox1F	5'-ACC GGT GTT TGG GAT TGT TA-3'
Drcox1F	5'-GTA TA TTT TGG GTT TAC ATA CTG TA-3'
Common reverse primer NTR	5'-ATA AGT ACG AGT ATC AAT ATC-3'

Table 1. Species-specific primer sets to amplify cox1 gene regions of different sizes.

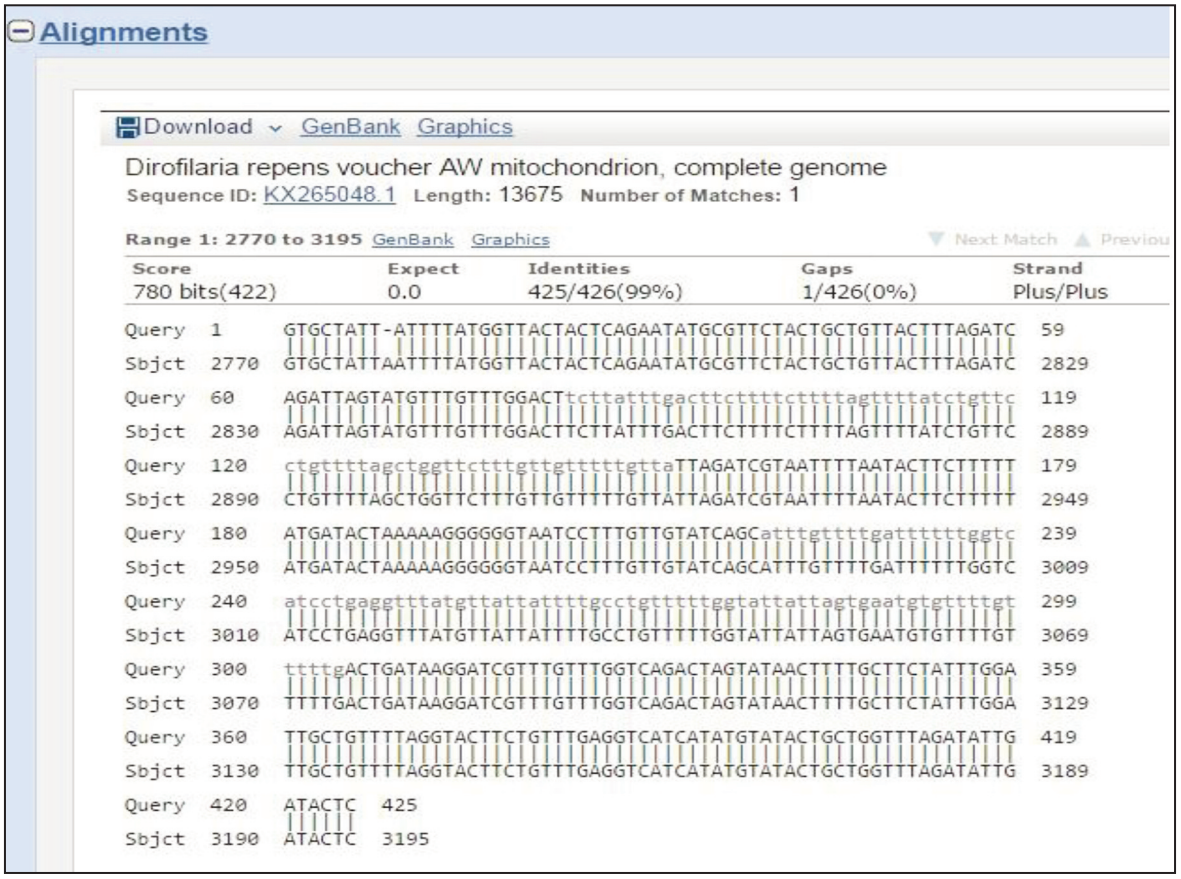

Figure 2. BLAST results.

analysis of the cox1 sequences produced here revealed from 99 to 100% identity to those available in GenBank™ (accession numbers DQ358814, KX 265048.1). The present study describes a rapid and accurate molecular method for the simultaneous detection of the most common filarioids infesting dogs and sometimes human patients.

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.

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 benignant forms of infection go unnoticed due to a lack of awareness among the medical profession. Cases of deep tissues localization usually evocate 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 dirofilariasis was diagnosed using molecular diagnostic test. In order to

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

Asimacopoulos P.J., Katras A. & Christie B., 1992. Pulmonary dirofilariasis. The largest single-hospital experience. *Chest*, 102: 851–855.

Bronshteĭn A.M., Supriaga V.G., Stavrovskiĭ B.I., Sabgaĭda T.P., Luchshev V.I., Korotkova G.I., Legon'kovIu A., Firsova R.A., Darchenkova N.N., Starkova T.V., Zhukova L.A., Bugrov A.N., 2003. Human dirofilariasis in the Moscow region. *Medici Parazitologie*, 3: 51–56.

Casiraghi M., Bazzocchi C., Mortarino M., Ottina E. & Genchi C., 2006. A simple molecular method for discriminating common filarial nematodes of dogs (Canisfamiliaris). *Veterinary Parasitology*, 141: 368–372. Epub 2006 Jul 20.

Chazan B., Scherbakov A., Kerner H. & Raz R., 2001. Autochthonous subcutaneous dirofilariasis in Israel. *Harefuah*, 140: 1125–1126.

Echeverri A., Long R.F., Check W. & Burnett C.M., 1999. Pulmonary diroflariasis. *Annals of Thoracic Surgery*, 67: 201e2

Fontanelli S.L., Gabrielli S., De Angelis M., Milardi G.L., Magnani C., Di Marco B., Taliani G. & Cancrini G., 2016. *Dirofilaria repens microfilariae* from a human node fine-needle aspirate: a case report. *BMC Infectious Diseases*, 16: 248.

Genchi C., Kramer L.H. & Rivasi F., 2011. Dirofilarial Infections in Europe. *Vector-Born and Zoonotic Diseases*, 11: 1307–1317.

Gioia G., Lecová L., Genchi M., Ferri E., Genchi C. & Mortarino M., 2010. Highly sensitive multiplex PCR for simultaneous detection and discrimination of Dirofilaria immitis and *Dirofilaria repens* in canine peripheral blood. *Veterinary Parasitology*, 172:160–163. doi: 10.1016/j.vetpar.2010.04.027. Epub 2010 Apr 28.

Nuchprayoon S., Junpee A., Poovorawan Y. & Scott A.L., 2005. Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. *American Journal of Tropical Medicine and Hygiene*, 73: 895–900.

Pampiglione S. & Rivasi F., 2000. Human dirofilariasis due to *Dirofilaria* (*Nochtiella*) *repens*: an update of world literature from 1995 to 2000. *Parassitologia*, 42: 231–254.

Pampiglione S., Canestri Trotti G. & Rivasi F., 1995. Human dirofilariasis due to *Dirofilaria* (*Nochtiella*) *repens*: a review of world literature. *Parassitologia*, 37: 149–193.

Pampiglione S., Rivasi F., Angeli G., Boldorini R., Incensati R.M., Pastormerlo M., Pavesi M. & Ramponi A., 2001. Dirofilariasis due to *Dirofilaria repens* in Italy, an emergent zoonosis: report of 60 new cases. *Histopathology*, 38: 344–354.

Raccurt C.P., 1999. Dirofilariasis, an emerging and underestimated zoonoses in France. *Medecine Tropicale*, 59: 389–400.

Rivasi F., Boldorini R., Criante P., Leutner M. & Pampiglione S., 2006. Detection of *Dirofilaria* (*Nochtiella*) *repens* DNA by polymerase chain reaction in embedded paraffin tissues from two human pulmonary locations. *Acta pathologica, microbiologica, et immunologica Scandinavica*, 114: 566–573.

Shibasaki M., Shito I., Konno A., Yusa T., Shibuya K., Kubosawa H. et al., 1997. A case of pulmonary dirofilariasis: cytological, pathological and immunological diagnosis. *Journal of the Japanese Society of Clinical Cytology*, 36: 174–178.