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Peri - Orbital Human Dirofilariosis: A Pitfall for Experimental ELISA

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SUMMARY

The dog nematodes *Dirofilaria immitis* (*D. immitis*) and *D. repens*, well known as zoonotic agents, can infect humans in whom they usually produce abortive infections or immature worms. Dirofilarioses, asymptomatic in most patients and suspected only when the worm reaches surface locations or imaging detects coin lesions, are underdiagnosed because both physicians do not consider this aetiology and the human immune response blocks the worm development at early larval stages, difficult to be located. The identification of the infecting species is based on morphological/genetical study of surgically removed specimens. Serology should be an alternative to the invasive methods, also useful to detect hidden infections; nevertheless, at present, there are no commercial kits to specifically diagnose human dirofilarioses.

The aim of the study was to test the sensitivity of experimental serological assays in a case of peri-ocular dirofilariosis caused by *D. repens*, which had been identified by morphology and molecular methods (PCR).

Serological investigations included two serum samples, one before surgery intervention and the other one six months after extirpating the worm. Specific antibodies against both somatic/metabolic antigens of *D. repens* and *D. immitis* adult specimens, and against antigens of the filarial endosymbionts belonging to the genus *Wolbachia* were evaluated. Sera were submitted to experimental enzyme immunoassay (ELISA) protocols, and to an ELISA commercial kit available to diagnose human tropical filarioses.

Diagnostic significant titres of specific antibodies by any applied test were not found.

As suspected, peri-ocular case of human infection due to *D. repens* proved a pitfall for serology, even if immunodiagnostic tests are designed with a wide range of antigens that are released by the worm, starting from its penetration in the human host.

***Key words: Dirofilaria immitis, Dirofilaria repens*, enzymeimmunoassay**

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INTRODUCTION

Dirofilaria immitis and *Dirofilaria* (*Nochtiella*) *repens* are aetiological agents of vector-borne diseases that usually affect domestic and wild carnivores. Humans act as accidental but increasingly frequent carrier of these filarial nematodes (1- 6) and only seldom allow them to reach the mature adult stage and to produce microfilariae in bloodstream circulation (3, 7).

Epidemiologically viewed, the prevalence of human dirofilarioses correlates to the prevalence of dog dirofilarioses, to the presence of „opportunistic" feeder mosquitoes species as vectors, and to the easy exposure of human population to bites of these vectors (1, 2). As documented by seroepidemiological surveys (8), human infections are more frequent than presented by the literature. Worms are mainly detected in subcutaneous and subconjunctival location, but lung location is described as well (2); moreover, most infections remain undetected due to the human immune response that blocks the worm development at larval stages (≥1 mm), difficult to be located. Finally, many cases are misdiagnosed, or not published, and further other are recovered spontaneously without medical intervention.

The identification of the aetiological agent of human infections has been, to date, based on morphological study, *in toto* or in histological sections, of surgically removed *Dirofilari*a specimens. Nevertheless, owing to the scarcity of diagnostic features in developing worms, this analysis leads to relatively easy identification only of adults removed before the degeneration process elicited by the inflammatory response of the host. Nowadays, molecular diagnostics are available, which allow correct identification even of a small share from the surgically removed parasite at any developmental stage (9, 10).

Serology should be an alternative to the invasive methods, also useful to detect hidden infections; nevertheless, at present, there are no commercial kits to specifically diagnose human dirofilarioses. It is only possible to use an experimental enzyme-immuno-assay (ELISA), designed as a result of studies that have identified specific polypeptides that permit the detection of specific antibodies against *D. immitis*, *D. repens* and against the filarial endosymbionts belonging to the genus *Wolbachia* (11- 13) that are present in the worm during all its life span, starting from the microfilaria to the male and female adult stages.

The aim of this paper was to test the sensitivity of these experimental ELISAs in one case of peri-ocular *D. repens*-filariosis reported in a patient living in Serbia.

MATERIAL AND METHODS

Two blood samples were drawn from the patient having *D. repens* infection, the first one before the surgery intervention, and the second six months after the worm removal. The nematode was located in the subcutaneous tissue of the periorbital region.

Parasitological analyses and morphometric characteristics obtained by television image analysis system Nikon Lucia M (Nikon, Japan) enabled us to identify the extirpated nematode as *D. repens*-like (the adult parasite was cylindrical, whitish, about 12 cm in length, 0.3 cm in width. Microscopic examination showed a thick cuticle (8 μm) with longitudinal ridges, 4 pairs of cephalic papillae, and a distance of 3.6 cm between the oral opening and the vulva. The worm was also processed by means of molecular tools (PCR with specific primers *D. repens*
(R1=5'GGTAAATCAAATGCCCAGAACCG3';
R2=5'CATCATTTCATGGTCTACCGG3') and *D. immitis* (I1=5'GGCTCACAGATGCTCAAA3';
I2=5'CCGTTACGCCGTTCATTCATTGCCGC3'), according to Favia et al. (9, 10), that made the final identification of *D. repens* (14).

Serum samples from the patient were analysed by means of tree ELISA protocols that use as antigens *D. repens* (DR) and *D. immitis* (DI) adult somatic/ metabolic polyproteins (11), and recombinant Wolbachia Surface Protein (rWSP), respectively. As positive and negative controls were used a pool of sera from three patients diagnosed as having subcutaneous dirofilariosis caused by *D. repens*, and pool of five sera from healthy individuals residing in an area free from canine dirofilariosis, respectively. Sera were tested against DR and DI somatic antigens, and rWSP in solid-phase ELISA at dilutions 1:10 and 1:50. The anti-human-IgG-peroxidase conjugate used (Dako, Glostrup, Denmark) was diluted to 1:5.000. Optical densities (OD) were measured in a plate reader at 492 nm.

Finally, the sera were submitted to an ELISA commercial kit available to diagnose human tropical filarioses.

RESULTS

Experimental ELISAs evidenced the level of the antibodies against *D. repens*, *D. immitis* and rWSP lower than the cut-off values in both sera, even if optical densities of the second serum proved lowered if compared to that shown by the serum taken before surgical intervention. The commercial kit gave the same negative results. Table 1 shows the OD evidenced.

Table 1. *Optical densities in two serum samples of patient with peri-orbital dirofilariosis*

	Optical densities (OD)			
	ELISA DR *	**ELISA DI ****	**ELISA rWSP *****	**Kit ELISA ******
Cut-off	0.8	0.9	0.3	0.6
Serum 1	0.48	0.30	0.21	0.42
Serum 2	0.42	0.19	0.19	0.44

* indirect enzymeimmunoassay with somatic/metabolic Dirofilaria repens antigens in solid phase
** indirect enzymeimmunoassay with somatic/metabolic Dirofilaria immitis antigens in solid phase
*** indirect enzymeimmunoassay with recombinant Wolbachia Surface Protein (rWSP) antigen in solid phase
**** indirect enzymeimmunoassay commercial kit available to diagnose human tropical filarioses

DISCUSSION

The detection and diagnosis of dirofilariosis in humans is difficult, due to the absence of bloodstream circulating microfilariae. Moreover, only subcutaneous nodules or superficial active worm migration usually urge the patient to seek medical attention, whereas internal nodules (pulmonary ones included) are detected accidentally, by imaging. Considering that both subcutaneous and pulmonary nodules can cause suspicion of malignant tumour or of other pathologies (tuberculosis, fungal infection), surgery is usually recommended (15).

Therefore, the surgically removed materials are the only start points for a possible dirofilariosis diagnosis, so that data about the *D. repens*- or *D. immitis*-infection prevalence have to be considered as unreliable (15). Then, histological identification of the worms (often pre-adults) found in nodules removed may be problematical because of the very few differential morphological parameters that can be analysed (less than the *in toto* worm observation), and also because of the disruption of the normal worm anatomy induced by the tissue response of the host. These constrains could hamper to identify dirofilarial worm at species level, and to ascribe some infections to other zoonotic species occasionally recognized in humans. As alternative, biomolecular techniques that reliably identify all species at any developmental stage could be now applied, but unfortunately, only in highly specialised laboratories. In any case, traditional morphology and innovative DNA analysis require previous removal of the worms, particularly invasive in case of its pulmonary location.

Serology could be an advantageous diagnostic alternative, which makes possible to avoid a surgical intervention that is always invasive. Moreover, this diagnostic tool can detect hidden infection and therefore offers a more reliable picture of the epidemiology of the zoonosis in any study area (11, 12, 15). However, this case of proven human infection was a pitfall for the diagnosis of subcutaneous dirofilariosis by experimental ELISAs: neither specific antibodies against somatic and metabolic antigens of *D. immitis* and *D. repens* adult worms nor against WSP proteins were evidenced. This is not surprising since antibody detection is directly related to the immune system reactivity of the host organism, to the location of parasite, and to the elapsed time from the moment of the worm penetration. The reason of these false negative results changes in the repertoire of surface antigens operated by the worm during its development, so that antigens of larval stages and adult worms are unlike. It has been supposed that specific antibodies against adult specific antigens can be detected when the parasite migrates for a long time through the body of the patient, since the immune system has the time to produce antibodies that are different from those immediately produced at the time when parasite penetrated. The immune system is elicited exactly from the L3 that penetrates the skin. After that, the L3 becomes L4, pre-adult, adult, and during its development becomes larger and continually changes its somatic and metabolic antigens, so that the immune response has to produce different antibodies. If the parasite directly reaches locations where antibodies have a difficulty to circulate (eye and nervous system), considering that the immune response is being formed exactly around the worm, antibodies are unable to reach the blood stream, and we are hampered to find them in serum samples. Instead, if the parasite migrates for a long time we have some chances to detect antibodies. This phenomenon is registered mainly for *D. repens* infections, probably because the developing worm migrates within the body without the direct contact with the blood. On the contrary, it seems that infections due to *D. immitis*, which enter the blood vessels during its development, offer more chances to detect antibodies in the blood. *Wolbachia* antigens released by the developing worm are the same during all its life, therefore could give more satisfactory results, but dirofilarial species harbouring *Wolbachia* and its migration without the direct contact with the blood is, evidently, the limiting factor (16). In the studied case, the worm was an immature female sized only 12cm in length (indeed not completely developed), and the patient did not complain of the previous worm migration phase. These

elements suggest that probably a short period of time elapsed from the infection to the worm detection in peri-ocular location, so hampering the development of any noticeable reactivity by means of the specific ELISAs developed, already proven weakly sensitive in such cases. It has been suggested that in such conditions the reactivity to the specific (26-40 kDa) polypeptides in western blot tests could be more efficient to diagnose the infection, even if ocular/peri-ocular cases recognize, with lower degree of intensity, only two groups of bands of approximately 35-37 and 39-40 kDa (11, 15, 17, 18). Unfortunately, currently, we cannot apply the western blot test.

CONCLUSION

In conclusion, ocular/peri-ocular cases of human infection due to *D. repens* are a pitfall for serology, even if designed with a wide range of antigens that are released by the worm starting from its penetration in the human host.

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EKSPERIMENTALNA ELISA NE DAJE ZADOVOLJAVAJUĆE REZULTATE U DIJAGNOSTICI PERI-ORBITALNE DIROFILARIOZE

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Sažetak

Vrste *D. repens* i *D. immitis,* paraziti pasa, dugo poznati kao uzročnici zoonoza, mogu kao nezrele jedinke da parazitiraju i u organizmu čoveka. Na dirofilarioze, najčešće asimptomatske infekcije, posumnja se u slučaju superficijalnih infekcija ili pri nalazu karakterističnih coin-lezija prilikom radiološkog pregleda pluća. Mnogi slučajevi humane dirofilarioze ostaju nedijagnostifikovani usled nerazmišljanja kliničara o mogućnosti razvoja ove parazitoze, kao i zbog imunske reaktivnosti koja stopira rast nematode. Identifikacija je moguća na osnovu morfoloških karakteristika ekstipiranog parazita kao i primenom metoda molekularne biologije. Serologija može biti alternativa invazivnoj dijagnostici, a može se koristiti u slučajevima „skrivenih" infekcija, ali do danas nema komercijalnih kitova za serološku dijagnozu dirofilarioza.

Cilj rada bio je ispitati osetljivost eksperimentalnog seološkog testa u dijagnostici peri-orbitalne dirofilarioze uzrokovane *D. repens* vrstom, koja je identifikovana parazitološkim i molekularnim metodama (PCR).

Eksperimentalnom imunoenzimskom metodom (ELISA) ispitana su dva uzorka seruma, jedan pre hirurške intervencije i drugi uzorkovan 6 meseci nakon ekstirpacije helminta. Ispitivano je prisustvo specifičnih antitela prema somatskim i metaboličkim antigenima adultnih formi vrsta *D. repens* i *D. immitis*, kao i prema površinskom *Wolbachia* antigenu (endosimbiont ovih filarija). Pored eksperimentalne ELISA metode, serumi su ispitivani i korišćenjem komercijalnog ELISA testa za dijagnostiku tropskih filarioza.

Nijednim primenjenim testom nisu utvrđena specifična antitela u dijagnostički značajnom titru.

Kao što se pretpostavilo, seološko ispitivanje u slučaju peri-orbitalne dirofilarioze izazvane vrstom *D. repens* ne daje zadovoljavajuće rezultate, čak ni prilikom primene širokog spektra antigena koje parazit ima ili produkuje u toku infekcije.

***Ključne reči: Dirofilaria immitis, Dirofilaria repens,* imunoenzimski test**