Canine cardiopulmonary nematodes in Morocco: Prevalence of Dirofilaria immitis and report of the first autochthonous infection with Angiostrongylus vasorum

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Dirofilaria immitis and *Angiostrongylus vasorum* are two of the most important, life-threatening cardiopulmonary nematodes affecting dogs. This study aimed to determine the current prevalence of *D. immitis* and to investigate, for the first time in Morocco, the occurrence of *A. vasorum* in dogs. Two hundred and thirteen blood and 291 fecal samples were collected from Moroccan dogs. Blood samples were examined using both the modified Knott's test and the in-clinic enzyme-linked immunosorbent assay, whereas fecal samples were examined using the Baermann technique. Fecal analysis revealed the presence of *A. vasorum* larva in one sample. This was the first reported case in a dog from Morocco. We believe that these preliminary results are of great interest to veterinary practitioners in Morocco, suggesting that veterinarians should consider *D. immitis,* but also *A. vasorum* in their differential diagnosis, and should perform regular screenings for these parasites.

Keywords: Cardiopulmonary nematodes, *Dirofilaria immitis*, *Angiostrongylus vasorum*, Dogs, Morocco

INTRODUCTION

Dirofilaria immitis and Angiostrongylus vasorum are two of the most important cardiopulmonary nematodes affecting dogs (Di Cesare et al., 2011). Dirofilaria immitis is a zoonotic vector-borne pathogen that is transmitted by several species of mosquitoes (Alho et al., 2018; Széll et al., 2020), while A. vasorum is transmitted to dogs by gastropod mollusks (Alho et al., 2018; Bowman, 2014). Adult stages of both nematodes reside in the right heart and pulmonary arteries of infected dogs (Alho et al., 2018; Di Cesare et al., 2011) and lead to serious and potentially fatal diseases (Del Prete et al., 2015; Di Cesare et al., 2011; Simón et al., 2012). Infected dogs may display cardio-pulmonary signs (e.g. exercise intolerance, ascites, tachycardia, cough, dyspnea, tachypnoea), neurological, ocular and gastrointestinal signs and, in the case of A. vasorum, coagulopathies can occur (Cardoso et al., 2012; Di Cesare et al., 2011; Maia et al., 2015; Del Prete et al., 2015; Tachmazidou et al., 2021).

Dirofilaria immitis has been identified throughout the world in tropical and temperate regions (Genchi and Kramer, 2020; Simón et al., 2012) while A. vasorum has been considered endemic in some isolated foci, including southern France, Denmark and the United Kingdom (Alho et al., 2018; Di Cesare et al., 2011). However, several factors such as urbanization, climatic changes and pet movements have led to the spread of these nematodes within endemic regions and their emergence in previously free areas (Di Cesare et al., 2011; Fuehrer et al., 2021; Genchi and Kramer, 2020; Morgan et al., 2021).

Cases of human infection with D. immitis have been reported in many areas of the world (e.g. USA, Australia, Japan) (Ziadi et al., 2005), but it is believed that the reported prevalence rates are underestimated due to undiagnosed or unnoticed cases, since the infection is usually asymptomatic (Alho et al., 2014; Simón et al., 2012). Humans may also develop overt disease presenting with cough, chest pain, hemoptysis, dyspnea, ocular conditions (Dantas-Torres and Otranto, 2020) and pulmonary nodules mimicking malignant lesions (Alho et al. 2018; Cardoso et al., 2012; Di Cesare et al. 2011). Thus, more damage is done by the physicians who perform unnecessary investigations, such as nodule biopsies.

Despite the clinical severity, the zoonotic potential and the endemicity of canine cardiopulmonary parasites in various Mediterranean countries (Carretón et al., 2020; Elhamiani Khatat et al., 2017; Mendoza-Roldan et al., 2020; Morelli et al., 2021; Pérez Pérez et al., 2021; Rjeibi et al., 2017; Sarali et al., 2020; Schnyder et al., 2017; Shukullari et al., 2015; Tahir et al., 2017), these nematodes are highly neglected in Morocco. No studies have previously been performed to assess the occurrence and prevalence of A. vasorum in Moroccan dogs, and published data on the prevalence of D. immitis are limited to the results of two studies performed in 1987 (Pandey et al., 1987) and 2017 (Elhamiani Khatat et al., 2017). This situation may lead to an underestimation of these parasites and probably to undiagnosed and improperly managed cases. Therefore, this preliminary study aimed to determine the current prevalence of D. immitis and to investigate, for the first time in Morocco, the occurrence of A. vasorum in dogs.

MATERIAL AND METHODS

Study population and location

Between January 2019 and March 2020, 213 blood and 291 fecal samples were collected from various Moroccan dog populations including owned, shelter, working and rural semi-stray dogs. Owned dogs were all collected at the Small Animal Clinic of the Hassan II Institute of Agronomy and Veterinary Medicine (IAV Hassan II), Rabat, Morocco. Shelter dogs were collected at shelters from the cities of Rabat, Marrakech, and Casablanca. Working dogs were recruited from a kennel located in Benslimane city and semi-stray dogs were sampled from the surroundings of Rabat and from the rural commune of Ait Ichou.

For each dog, a detailed signalment and history (Breed, Age, Sex, dog’s role or activity, vaccination status, and medical history) were obtained from the owners (owned and semi stray dogs), the shelters supervisors (shelter and breeding dogs) or the dog trainers (working dogs). Blood samples were not collected from dogs younger than 6 months because of the life cycle and the prepatent period of D. immitis (Alho et al., 2014).

All dogs underwent a physical examination and they were considered symptomatic when they displayed respiratory, cardiac, hemorrhagic, ocular or neurological disorders that could not be associated with other diseases (Cardoso et al., 2012; Conboy, 2009; Ducos de Lahitte and Ducos de Lahitte, 2009; Liu et al., 2017; Tachmazidou et al., 2021). In addition, thoracic radiographs and echocardiography were performed in five D. immitis positive dogs.

Before including dogs in this study, informed consent was obtained orally from the owners (owned and rural semi-stray dogs) and from the persons in charge of the rescue shelter (shelter dogs) and the working dogs' kennel (working dogs).

Sample collection

For each of the 213 dogs, 5 ml of whole blood were collected from the cephalic or jugular vein. Blood samples were divided into a tube without anticoagulant and a tube with EDTA. Serum was separated by centrifugation then frozen at -20°C until tested with the in-clinic enzyme-linked immunosorbent assay kit (SNAP 4DX Plus, IDEXX Laboratories, Inc., Westbrook, ME) for the detection of D. immitis antigens. EDTA samples were stored at 4°C for a maximum of two days during which the modified Knott's technique was performed.

In addition, fresh fecal samples were collected from 291 dogs into a sterile container and the Baermann technique was performed within the day of sampling.

Sample analysis

Modified Knott's technique

The modified Knott's technique was performed to concentrate and identify microfilariae. Nine ml of 2% formalin were mixed with 1 ml of EDTA blood sample, then centrifuged for 5 minutes at 2000 RPMs. The supernatant was poured off and one drop of 0.1% methylene blue was mixed with the sediment. Drops of the sediment were placed on a microscope slide and covered with a coverslip. The entire slide was examined under a light microscope with 100x and 400x magnifications (Di Cesare et al., 2011; Zajac and Conboy, 2013). Microfilariae were identified using a specific key of identification (Zajac and Conboy, 2013).

Enzyme-linked immunosorbent assay (ELISA)

Dirofilaria immitis antigens were detected using a commercial in-clinic enzyme-linked immunosorbent assay (ELISA), SNAP 4DX Plus (IDEXX Laboratories, Inc., Westbrook, ME), according to the manufacturer's directions. The test ensures a sensitivity and specificity of 98.9% and 99.3%, respectively (Stillman et al., 2014).

Baermann technique

The Baermann technique was performed as previously described by Hinney et al. (2017) to isolate A. vasorum larvae (Traversa et al., 2010). Briefly, 5 g of feces were covered in gauze, placed in a tea strainer in the funnel of the Baermann apparatus, and submerged in water. After 24 hours, drops of water were collected from the bottom of the tube and examined under a light microscope with 100x magnification. Larvae were identified using a specific key of identification (Zajac and Conboy, 2013).

Statistical analysis

The relationship between prevalence and risk factors was evaluated by the Fisher's exact test.

RESULTS AND DISCUSSION

Blood testing

Blood samples were collected from four dog categories, including owned (n= 37, 17.4%), working (n= 55, 25.8%), shelter (n= 93, 43.7%) and rural semi-stray dogs (n= 28, 13.1%). Two age groups were distinguished comprising young adults (6-12 months) (n= 26, 12.2%) and adults (>1 year old) (n = 187, 87.8%). Males and females represented 54.0% (n= 115) and 46.0% (n= 98) of the studied population, respectively. Eighty-three dogs (38.7%) were asymptomatic and 130 (61.0%) were symptomatic.

Dirofilaria immitis antigens were detected in 10 dogs (4.7%). Microfilariae were detected in all dogs that were positive to serology (Figure 1), except in one case. This was probably due to an amicrofilaremic infection (Borthakur et al., 2015; Simón et al., 2012).

The Dirofilaria immitis prevalence revealed in our study (4.7%) was lower than the prevalence reported in two previous studies conducted in Morocco by Pandey et al. (1987) (12.3%) and by Elhamiani Khatat et al. (2017) (16.1%). However, it should be considered that the study conducted by Pandey et al. (1987) involved exclusively stray dogs, mostly from rural areas, who are known to be more frequently exposed to vectors and other environment factors, and do not receive preventive treatments (Elhamiani Khatat et al., 2017; Kunathasan Chelliah and Šlapeta, 2019). Also, the presence of D. immitis worms was determined by necropsy, which is a gold standard test (Ranjbar-Ba et al., 2007). Similarly, in the study conducted by Elhamiani Khatat et al. (2017), in most samples, significantly higher seropositive rates were from rural free-living dogs that never received preventive treatments. In our study, only a few samples were from rural dogs (28/213 dogs), and most of them were regularly treated with permethrin, which may have limited their exposure to infected mosquitoes (Alho et al., 2018; ESCCAP, 2021). Additionally, false-negative results cannot be excluded in our study, in the case of prepatent infections, single male infections or infertile female worm infections (Vieira et al., 2014). A reduction in the prevalence could also be due to increased awareness of veterinary practitioners and owners (Genchi and Kramer, 2020), probably leading to a wide use of prophylactic treatment. Therefore, we cannot determine if the reported prevalence is related to the samples and/or diagnostic biases or if it is a real decrease in the current prevalence of D. immitis in Morocco. More detailed epidemiological and longitudinal studies are necessary to confirm these findings and inform on potential prevalence changes (Pérez Pérez et al., 2021).

The prevalences of D. immitis reported in other Mediterranean countries varied from 1.4% to 14.5% (Esteban-Mendoza et al., 2020; Pantchev et al., 2009; Pérez Pérez et al., 2021; Rjeibi et al., 2017; Sarali et al., 2020; Schüle et al., 2015; Selim et al., 2021; Tahir et al., 2017). In general, the wide variation of D. immitis prevalence across different countries is related to the heterogenicity of studies design, including different study areas (ecological and geographical factors), dog populations (e.g. activity, age, sex, breed) and diagnostic methods (antigen serology, blood smear examination or molecular methods) (Genchi and Kramer, 2020; Sarali et al., 2020).

This study showed that owned dogs had the highest prevalence level of D. immitis infection, followed by working and shelter dogs, while none of the rural semi-stray dogs was found positive. Adults were more infected than young adults and the prevalence was higher in males compared to females (Table 1). Despite these differences, dog activity ($p = 0.2000$), age ($p = 0.6145$) and sex ($p = 0.3481$) were not statistically significant risk factors. However, this result may have been influenced by the low prevalence in our study.

In many previous studies, statistically significant differences were reported between various dog categories, with higher infection rates reported in outdoor compared to indoor dogs (Borthakur et al., 2015; Cancrini et al., 2000; Kunathasan Chelliah and Šlapeta, 2019; Omar et al., 2018; Pérez Pérez et al., 2021). However, in our study, most of these dogs were regularly treated with external antiparasitic drugs which may have limited their exposure to mosquitoes, resulting in the low reported infection rates (Alho et al., 2018; ESCCAP, 2021). The absence of positive rural dogs in our study was probably related to the low number of samples from this dog category, rather than to a real absence of infection.

Although not statistically significant, it is noteworthy that infection rates were found higher in adults and male dogs. Several previous papers reported similar findings (Atas et al., 2018; Elhamiani Khatat et al., 2017; Omar et al., 2018; Pérez Pérez et al., 2021; Sarali et al., 2020). There is evidence indicating that the chance of getting infected with D. immitis increases with age (Sarali et al., 2020), probably due to the longer exposure of adult dogs to mosquito bites (Hou et al., 2011; Maia et al., 2015). The higher prevalence in males may be assigned to their lifestyle since they are kept outdoor more often (Atas et al., 2018; Sarali et al., 2020).

Our study showed that the prevalence of D. immitis was significantly higher in symptomatic dogs compared to asymptomatic ones ($p = 0.0073$). This is in line with some reports revealing higher infection rates in clinically suspect dogs compared to healthy ones (Cardoso et al., 2012; Hesselink, 1988), in contrast to one study where the prevalence was higher in asymptomatic dogs (Elhamiani Khatat et al., 2017). The presence or absence of clinical signs may be related to the number of worms harbored by the infected dogs, as described by Hou et al. (2011). Clinical signs displayed by symptomatic dogs of this study, are provided in Table 2.

All the dogs that were positive for D. immitis and subjected to medical imaging showed at least one abnormality on the thoracic radiography. This is in accordance with published studies which report that radiographic abnormalities are present in approximately 85% of dogs with heartworm infection (Bowman and Atkins, 2009). Reported radiographic signs mainly included enlarged and/or tortuous pulmonary arteries (5/5 dogs), enlargement of the right side of the heart (4/5 dogs) and pulmonary parenchymal lesions (4/5 dogs) (Figure 2) (Bowman and Atkins, 2009; Reifur et al., 2004).

Dirofilaria immitis worms (parallel linear bands) were identified in the right ventricle of three dogs among the five subjected to echocardiography (Figure 3). According to literature, heartworms are only rarely demonstrated in ultrasounds except in dogs with caval syndrome or heavy worm burdens (Bowman and Atkins, 2009).

Fecal samples testing

Fecal samples were collected from 96 owned (33.0%), 139 shelter (47.8%) and 56 working (19.2%) dogs. Three age groups were distinguished including puppies (n= 70, 24.0%), young adults (n= 31, 10.6%) and adults (n= 190, 65.3%). There were 126 males (60.9%) and 81 females (39.1%). Sex of the remaining 84 dogs was not recorded because of logistical issues.

Our study revealed the occurrence of a single A. vasorum larva in one fecal sample (0.3%). The larva was 400 µm in length and the tail showed an S-shaped curve and a dorsal spine (Figure 4). This fecal sample belonged to a 6 month-old, male, asymptomatic owned Malinois Belgian Shepherd. This dog was sampled in Rabat, but the owner said he had bought it from the north of Morocco (Tangier).

This is the first autochthonous case of A. vasorum reported in a dog from Morocco. This finding may be a proof of the expanding range of the parasite. Geographical expansion of A. vasorum in previously nonendemic areas has been documented in various reports (Fuehrer et al., 2021; Hurníková et al., 2013) and autochthonous cases have been reported in several previously free countries such as the Netherlands, Hungary and Slovakia (Hurníková et al., 2013; Majoros et al., 2010; van Doorn et al., 2009). The reason for this expansion is not clearly explained but some hypotheses have been suggested including climate-change, diffusion of gastropods, increased dog movement and urbanization of red foxes (De Zan et al., 2021; Fuehrer et al., 2021; Hurníková et al., 2013; Morgan et al., 2021).

However, it should be considered that although the Baermann’s technique used in our study is a gold standard for the diagnosis of angiostrongylosis, this method has some limitations and cannot detect infections during prepatent period, in the case of low worm burdens and intermittent larval excretion, or when feces are tested late after their excretion (containing inactive or dead larvae)

(Liu et al., 2017; Schnyder et al., 2014; Simin et al., 2014). Currently, rapid in-clinic serological tests for the detection of A. vasorum antigens (AngioDetect™, IDEXX) or antibodies (Elisa and Western Blot) are available, allowing an easy and quick diagnosis. PCR can also be performed for the detection of the parasite DNA. Yet, none of these technics is currently available in Morocco. This highlights the need of introducing these tests in the country, particularly the AngioDetect test, to overcome the Baermann limitations and to enable veterinarians to confirm the diagnosis (Schnyder et al., 2014). It is also recommended to perform further studies using more precise techniques, such as serological tests, to confirm our result and to define if the reported autochthonous case of A. vasorum is a sporadic case or if this parasite is well established and more widespread in Morocco.

CONCLUSION

This study confirmed the occurrence of D. immitis in Moroccan dogs and reported, for the first time in the country, a case of infection with A. vasorum. These results suggest that veterinarians in Morocco should include cardiopulmonary dirofilariasis and angiostrongylosis in the differential diagnosis of dogs displaying cardiopulmonary signs, bleeding, neurological, ocular, compatible radiographic and/or echocardiographic signs. This will help to avoid undiagnosed cases and ensure an early diagnosis which may improve the prognosis. In this context, it is also recommended to perform screening for these cardiopulmonary. Finally, considering the lack of epidemiological data regarding canine cardiopulmonary parasites in Morocco, further larger studies are highly needed, using better diagnostic tools (direct microfilaria detection, Baermann, serology and PCR), to provide more information on these diseases and to contribute to the establishment of efficient and adapted control and prevention strategies.

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