Dirofilaria immitis infestation in imported police (K-9) dogs in Iraq: clinicopathological and molecular investigations study

Infecção por *Dirofilaria immitis* em cães policiais importados no Iraque: estudo clínico-patológico e de investigações moleculares

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ABSTRACT

Dirofilaria immitis, the cause of heartworm infestation (HWI) or dirofilariasis, affects members of the Canidae and remains a worldwide clinical problem. In Iraq, dirofilariasis was believed absent until 2009, when the Karbala Governorate was reported as an endemic area for canine dirofilariasis. Consequently, this study intended to investigate the occurrence of *Dirofilaria immitis* in police dogs in one police academy in Iraq and to study the gross and histopathological changes in 5 dead dogs, as well as to identify the species of the causative parasite using PCR technique. Thirty-nine police dogs, aged between 6 months and 12 years were included in this study. For the microfilariae investigation, 5 ml blood samples were collected from all dogs in EDTA tubes and examined by Knott's method. The systemic necropsy performed in five dead dogs showed severe clinical signs of dirofilariasis and tissue specimens were sent for routine histopathological processing. For the molecular analysis, adult worms of the detected *Dirofilaria spp.* were used for DNA extraction and amplification of the *cox1* gene. Fifteen of 39 (38.46%) dogs were diagnosed with moderate to severe microfilariasis. The dead dogs revealed typical severe clinical signs of dirofilariasis. Moreover, typical gross and histopathological changes were also seen, accompanied by generalized thromboembolic lesions, suggesting the occurrence of the caval syndrome. The PCR investigation confirmed that *D. immitis* was the species present in Iraq. In conclusion, this study establishes that Iraq is a newly reported endemic area for dirofilariasis. Moreover, the infestation occurring in these cases most probably happened inside Iraq. The authors recommend doing further epidemiological studies concerning the occurrence of *D. immitis* in local dogs as well as in the imported dogs in all Iraqi governorates to better understand the epidemiological map of this disease and to introduce an active treatment and preventive program. Awareness and education regarding this disease should be provided to the veterinarians, dog guiders and people in direct contact with dogs, as this disease is one of the important zoonotic diseases.

Keywords: *Cox1* gene. *D. immitis*. Iraq. K-9. Microfilariae.

RESUMO

A *Dirofilaria immitis*, causadora da infestação pelo verme do coração (IVC) ou dirofilariose afeta os membros da família Canidae e ainda é um problema clínico mundial. Até o ano de 2009, acreditava-se que o Iraque fosse livre da dirofilariose, porém nessa ocasião a governadoria de Kerbala foi relatada como uma área endêmica de dirofilariose. Assim, o presente trabalho foi realizado para investigar a ocorrência da *Dirofilaria immitis* em cães policiais em uma academia de polícia do Iraque, estudar as alterações macroscópicas e histopatológicas em cinco cães mortos, bem como, identificar as espécies do parasita causador com o emprego da técnida de PCR. Trinta e nove cães policiais com 6 meses a 12 anos de idade foram incluídos no estudo. Amostras de sangue de cinco mililitros foram colhidas por animal, em tubos com EDTA e foram examinadas pelo método de Knott. A necropsia sistêmica foi realizada em cinco cães que haviam apresentado sinais clínicos severos de dirofilariose e espécimens dos seus tecidos foram enviados para o processamento histopatológico de rotina. Para a análise molecular dos vermes adultos de *Dirofilaria* spp, foi empregada a extração do DNA e a amplificação do gene *cox1*. Quinze de 39(38,46%) cães foram diagnosticados com uma microfilariase variável de moderada para severa. As alterações macroscópicas e histopatológicas foram acompanhadas por lesões generalizadas tromboembólicas sugestivas da ocorrência da síndrome da veia cava. A investigação de PCR confirmou que a *D.immitis* era a espécie presente no Iraque. A conclusão do estudo estabeleceu que o Iraque deve passar a ser considerado como uma nova área endêmica da dirofilariose. Além da infestação registrada nos casos descritos é provável que ela também

esteja presente em outras regiões do Iraque. Os autores recomendam a realização de estudos epidemiológicos para investigar a ocorrência de *D.immitis* tanto nos cães locais bem como em cães importados em todas as governadorias do Iraque, para ser construído o mapa epidemiológico da distribuição da doença e implantadas as ações de tratamento e de um progrma preventivo. Ações de educação em saúde sobre a doença deverão ser dirigidas para os veterinários, tratadores de cães e para pessoas em geral que tenham contato com os cães, pois esta doença é uma importante zoonose.
Palavras-chave: Gene *cox1*. *D.immitis*. Iraq. K-9. Microfilarias.

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Introduction

Dirofilariasis or heartworm disease is a severe and potentially fatal disease of dogs. It is caused by a blood-born parasite called *D. immitis* (Ceribasi & Simsek, 2012) and belongs to the subfamily Dirofilariinae. It has been identified as the most significant parasite species of the right ventricle and pulmonary artery of domestic dogs and wild canids. It is also infests felines and humans. The disease is transmitted by Culicide species and *Anopheles spp;*, moreover, it is distributed around the globe in tropical, subtropical and temperate areas. Nevertheless, its circulation is significant in areas with vast mosquito numbers (Mccall et al., 2008a). Transmission of *D. immitis* is reliant on three main factors: a suitable ambient temperature; the presence of competent mosquito vectors; and a large available outdoor canine population (Mccall et al., 2008a,b). Vascular, pulmonary and renal damage is elicited primarily in severe and chronic cases that lead to the death of the parasitized animals with or without treatment.

The disease is considered as one of the zoonotic diseases, since the vectors are anthropophilic and the existence of dirofilaric dogs is of significant public health importance, particularly in warm climate countries (Simonsen et al., 2014). The infected dogs serve as reservoirs and provide blood meal containing microfilariae for mosquitoes. Inside the mosquito mid-gut, the microfilariae go through a series of changes and develop the infective larvae (L3) that migrate through the Malpighian tubules to the head of the

mosquito and are transmitted to a new host, whether human, dog, or other mammal, when it takes its next blood meal (Mccall et al., 2008a; Simonsen et al., 2014; Simon et al., 2009; Genchi et al., 2007).

Dogs may live in close proximity to their owners, often sleeping within the same room and travelling with them, and therefore having shared exposure to household and recreational risk factors, whereby the health of the dog may mirror that of their owner (Schmidt, 2009). However, humans are less suitable hosts, in which the parasite usually cannot complete its life cycle (Fuehrer et al., 2016) and the dead *D. immitis* lodge in the peripheral pulmonary artery, causing a small and spherical infarction. This lesion appears as an occasional disease in the lungs that appear as solitary non-calcified coin lesion discovered on a routine chest radiograph (Simón et al., 2012).

Dirofilariasis is considered as an emerging disease attributed to the global weather change caused by human interference in the environment, in addition to improper pet management and the presence of wild reservoirs (Genchi et al., 2007; Simón et al., 2012).

The initial injury in the heartworm infestation arises in the pulmonary arteries and lungs. The severity of damage depends on the numbers of worms, length of disease, and host's reaction to the existence of parasites. It is established that the L5 heartworms elicit the damage when they arrive in the pulmonary artery after three months of infestation (Atkins, 2005). The vascular damage and lung disease accompanied with signs of respiratory disease are initiated by the immature adult worms due to eosinophilia, and eosinophilic infiltrates.

Meanwhile, the adult worms classically live in the caudal pulmonary vascular tree and produce further damage through the releasing of toxic products and the host's specific immunological reaction to these products, in addition to mechanical trauma. The earlier vascular changes include endothelial damage and desquamation, villous proliferation, activation and attraction of platelets and leukocytes. All these events may finally produce muscle cell proliferation and collagen accumulation, causing fibrosis.

However, the worms dead due to treatment cause the most severe damage, including thrombosis, granulomatous inflammation and villous inflammation. Furthermore, blood vessels may become thrombosed, thickened, widened,

convoluted, noncompliant, and nonfunctional. Heartworms produce vasoactive elements that elicit vasoconstriction and hypoxia, which initiate pulmonary hypertension and compromised cardiac production (Kitoh et al., 2001). The pressure overload of the right ventricle occurs from pulmonary hypertension, subsequent in compensatory, concentric ventricular hypertrophy. However, in severe cases that occur in high worm infestation or chronic infestations, chronic pulmonary hypertension with tricuspid insufficiency results in elevated cardiac filling pressures and congestive heart failure. Thromboembolism may cause acute decompensation by producing or provoking pulmonary hypertension, right heart failure, or pulmonary infarction. Therefore, dead worms tend to worsen the vascular damage and enhance coagulation.

In Iraq, canine dirofilariasis was believed to be absent from the country. Nonetheless, in 2009, dog heartworm disease was first reported in Karbala Governorate, and adult *D. immitis* were found in the hearts of 16 of 22 examined dogs at 73% (Amall et al., 2009). Moreover, the authors assumed that heartworm disease is usually present but not diagnosed previously, or it is imported from canine dirofilariasis endemic countries (Amall et al., 2009).

In 2011, renal and myopathy lesions of *D. immitis* in naturally infested dogs has also been reported in Al-Hindya district / Karbala Governorate in 98 infested dogs of 457 animals (Enaam et al., 2011). The military working dogs (MWDs/ K-9) are used internationally to help countries in protection and safety tasks. In Iraq, after the 2003 war, K-9s have been extensively used to help reinforce security and investigate explosive devices.

Since 2003, a large number of police and MWDs have been imported to Iraq from different countries by interior and defense ministries. These dogs are divided into different police dog academies in all Iraqi governorates and according to the security demand (Al Salihi et al., 2017).

Information on the extent and distribution of canine parasites in general and *D. immitis* in particular in working police dogs in Iraq is absent, as these dogs are under parasitic prevention. Consequently, the present study aimed to determine the occurrence of *D. immitis* in K-9 dogs in one of the police academies in Iraq, to study the gross and histopathological changes in 5 dead dogs, and to identify the species of causative parasite using PCR technique.

Materials and Methods

Animals

This study was approved by the animal ethical and research committees of Al Muthanna University/ College of veterinary medicine. Study population consisted of 39 police dogs, ages ranging from 6 months to 12 years. EDTA (ethylenediaminetetraacetic acid) tubes were used to collect blood samples from the cephalic vein of all dogs by the Knott's method. Briefly, one ml of each blood sample was mixed with 9 ml of 2% formaldehyde, then was thoroughly shaken for RBC hemolysis. After centrifugation, Giemsa stain was added and the mixture was then examined under a light microscope to detect any possible microfilariae (Newton & Wright, 1956).

Five dogs showed severe clinical signs, including appetite and weight loss, coughing with severe respiratory distress, hemoglobinuria, bloody vomiting, and diarrhea, severe dehydration, weakness, and died suddenly. All dead dogs were referred to Al Muthanna University / College of Veterinary Medicine / Department of Pathology for postmortem examination. The systemic necropsy was performed for all dead dogs and all carcasses.

All organs from each dog, including heart, lungs, liver, spleen, kidney, blood vessels, eyes, testis, brain, stomach, intestine, pancreas and pieces of the skin were collected and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin and examined with light microscope connected with an image analyzer and camera. Each specimen of adult worm was extensively washed in physiological saline after removal from the heart and pulmonary arteries and preserved in 70% (v/v) ethanol until extraction of genomic DNA.

Molecular analysis

For DNA extraction, the adult worms of *Dirofilaria* preserved in 70% ethanol were used. At the time of extraction, *Dirofilaria* adult worms were thoroughly washed twice in distilled water to remove ethanol. The total genomic DNA was extracted using the DNA Extraction Mini Kit (GeneAll, Korea). According to the instruction of the manufacturer, DNAs were eluted and stored at -20 °C until PCR amplification. PCR amplification was done according to the method described previously by Murata et al. (2003). The cytochrome oxidase I (COI) (*cox1*) gene in the mitochondrial DNA of the worm was examined because the sequence from several nematode species is known. A 656- nucleotide fragment of COI region was amplified by the polymerase chain reaction (PCR) with primers COI intF (5'- TGATTGGTGGTTTTGGTAA-3') and COI intR (5'- ATAAGTACGAGTATCAATATC-3') (Murata et al., 2003). A PCR final reaction volume of 25 μl, consisting of 12.5 μl of PCR mix (2x Master Mix RED Ampliqon, Denmark), that contained 0.5 μM of dNTPs, 1.25 U Taq DNA polymerase, and 1.5 mM $MgCl_2$; 25 pmol of each primer, and five μl

of template DNA were performed. The cycling conditions were: 1 cycle of 94 °C for 5 min (as primary denaturation), subsequently, 30 cycles of 94°C for 30 sec (denaturation), 52 °C for 45 sec (annealing), and 72°C for 60 sec (extension), and followed by a final extension step of 72 °C for 7 min. A negative control sample was included in each run and comprising water instead of template DNA. The PCR products were examined utilizing a 1.5% TBE (Tris 0.09M, Borate 0.09M, EDTA 0.02M) agarose gel and subsequently stained with FluoroDye Fluorescent DNA Loading Dye for loading and determining DNA markers (SMOBiO DM3100). Electrophoresis employed 90 V for 1 h. The PCR products were examined using a UV Transilluminator (UVItec, EEC) and digitally photographed. According to the instructions of the manufacturer, AccuPrep Gel purification kit (Bioneer, Korea) was used to purify the amplified PCR product representing the *cox1* gene. Unidirectional sequencing was done in the PCR for purified products using the forward primer. Then, the nucleotide sequences were compared with genic sequences deposited in Genbank (http:// www. ncbi.nlm.nih.gov/).

Results

Blood parasitological survey

Out of 39 examined police dogs, 15 (38.46%) were diagnosed with moderate to severe microfilaremic *D. immitis,* detected from prepared smear and Knott's test after adding Giemsa stain.

Gross pathology findings

The systemic necropsy of the five dead dogs revealed virtually similar gross pathological lesions.

The subcutaneous tissues were severely congested with hemorrhagic patches (Figure 1). All capillaries were engorged, and its blood contents revealed gray-white coagulated materials that appear along the clotted blood (Figure 1).

The abdominal cavities of 2 dogs revealed the presence of serosanguinous fluid about 50 to 300 milliliters in volume (Figure 2). All organs of the abdominal cavity were severely congested in all dogs. Moreover, a large amount of straw-colored pleural fluids was also found in other dogs.

The lungs revealed different gross lesions including severe diffuse dark hemorrhage and edematous areas, congestion and gray collapsed areas with a surrounding zone of inflammatory reaction in the dorsal and ventral

Figure 1 - Dog necropsy with heartworm disease, thoracic incision. Severely congested subcutaneous tissues and vessels engorgement.

Figure 2 - Dog necropsy with dirofilariasis - abdominal cavities. Presence of serosanguinous fluid in the abdominal cavity in case 1 (Right) & straw-colored pleural fluids case 5 (Left).

aspects of the lungs. Emphysematous areas were found and distributed through the lungs lobes accompanied with pleural collapse (Figure 3).

The main pulmonary artery and both left and right branches appeared as wrinkled and thickened intima and, when open, contained remnants of dead worms surrounded by thrombi (Figure 4).

The filariae were located in both right and left pulmonary arteries. A severe diffuse dark hemorrhage and edema extended around the right caudal artery, where the lumen was occupied by extended thromboembolism that surrounded the dead worms. On cross section, the worm was tightly crowded into the lumen of the vessel and was associated with thrombosis. The left pulmonary arteries also revealed hemorrhage around it, and thrombosis packed a worm inside the lumen. Moreover, gross lesions were found in the heart that revealed dilation of the right atria and ventriculi with severe congestion with a large thromboembolic formation attached over the atrioventricular valve and involving the pulmonary valve and pulmonary artery. The thrombus was completely occluding the atrium and ventriculus and the pulmonic valve (Figure 5).
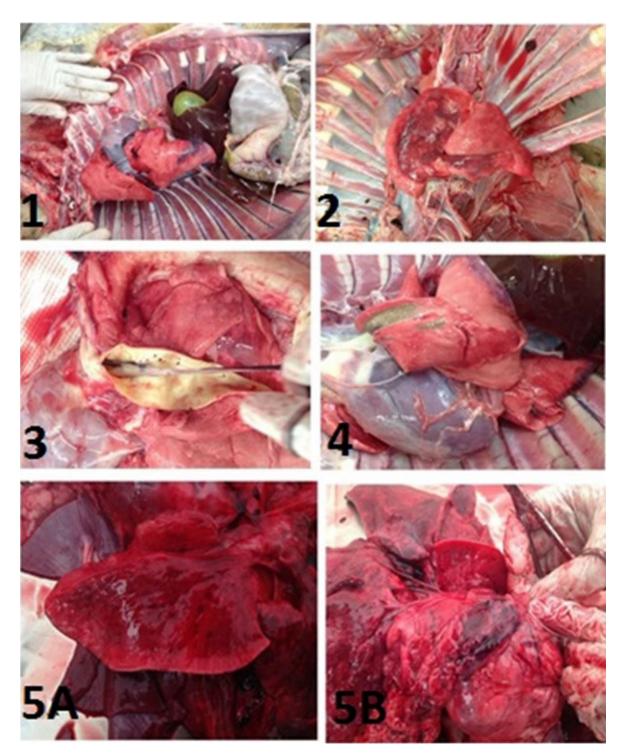

Figure 3 - Dogs necropsy with dirofilariasis. The different gross pathological lesions of lungs in cases 1, 2, 3, 4 and case 5 (A&B).
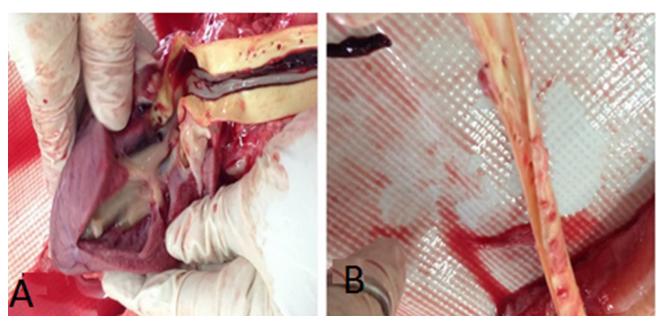
Figure 4 - Dog necropsy with dirofilariasis - lungs. A. the thrombus formation in the right side of the heart that extended to the artery, B. wrinkled and thickened artery.
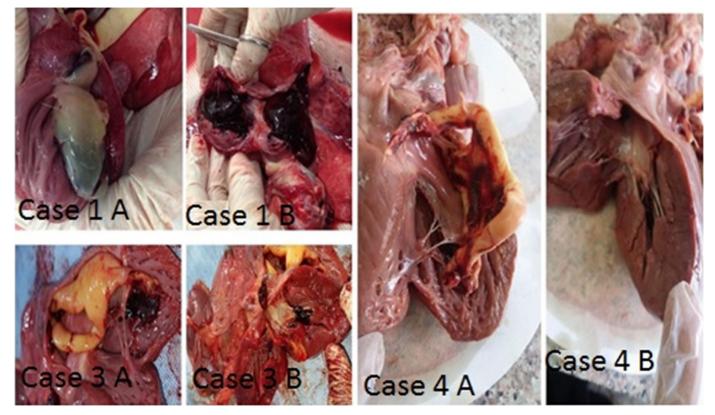

Figure 5 - Dog necropsy with dirofilariasis - lungs. Thrombus occlusion, occupying the right (A) left (B) side of the heart in cases 1, 3 & 4.

The left side of the heart revealed the same lesions, but in less degree. In all necropsied dogs, the liver was enlarged in size, congested as dark blackish red, showing stasis and a visible lobular pattern. Moreover, the kidneys revealed variable sizes and were increased in size, with a brown coloring (Figure 6).

Moreover, severe congestion, petechiae and ecchymosis was noted in the urinary bladder in all dogs. The bladder was also markedly distended with thickened walls. The mucosal surface of the bladder exhibited irregular, well-demarcated, raised yellow-tan areas intermixed with depressed hemorrhagic areas. All bladders were contained turbid pinkish gray urine with mucus consistency. All parts of the digestive tract were severely congested, accompanied by the severely congested pancreas.

Histopathological changes

The microscopic examination of different organs sections of the five dead dogs revealed diffuse alterations and microfilariae found throughout the vessels of different organs, including lungs, liver, kidneys, heart, and spleen (Figure 7).

The vascular lesions were the most significant lesion observed in the lungs. The intima of the pulmonary arteries comprised thickening with the formation of raised blunted folds prominent above the surface composed of a single layer of endothelial cells. Eosinophils, lymphocytes, macrophages and plasma cells were infiltrated in multiple

Figure 6 - Dog necropsy with dirofilariasis. Gross pathological changes in livers and kidneys in cases 1, 3 & 4.
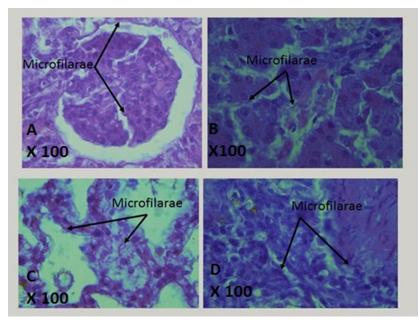

Figure 7 - Microscopic examination of dog with dirofilariasis. Presence of microfilariae throughout the vessels kidneys (A), liver (B), lungs (C) and spleen (D).

areas of the intima accompanied by sections of adult *Dirofilaria* that were frequently observed in larger branches of the pulmonary arteries.

The intima frequently revealed extensive thickening that resulted in the formation of observable villous-like bodies (villous endarteritis) that projected into the lumen and obliterated the affected vessels. A marked pulmonary emphysema, congestion, and edema accompanied by marked bronchial smooth muscle hypertrophy were also seen. Microfilariae were observed in the lungs of all dogs. The lung sections also revealed multifocal accumulation of neutrophils, macrophages, and eosinophils. Some lung sections also revealed focal thickening of alveolar walls and interstitial pneumonia (Figure 8). Pulmonary arteries thrombosis was present in all dogs.

Moreover, lymphoid cuffing of bronchi, bronchioles, and vessels were also prominent. Sections of the heart revealed a valvular thrombus containing large numbers of mononuclear inflammatory cells and eosinophils surrounding dead heartworms. Additionally, the sections of the mature thrombus revealed collagenous network interwoven with dead heartworms and surrounded by mononuclear and polymorph nuclear cells. Some heart sections also revealed focal lymphocytic myocarditis.

Liver sections revealed chronic passive congestion and the presence of microfilaria between liver sinusoids. Dystrophic changes, hydropic hepatosis with hyperhydrated hepatocytes, and pyknosis or karyopicnosis were other prominent histopathological changes accompanied by venous and lymphatic stasis and perivenous edema.

Sections of the kidneys from all dogs revealed marked pathological changes and the microfilaria found in the capillaries of the enlarged glomeruli accompanied by thickened capillary walls. Chronic membrano-proliferative glomerulonephritis, interstitial nephritis with multiple cortical and medullary damaging were obvious and accompanied by infiltration of histiocytes, plasma cells, and lymphocytes. Fibrosis and infiltration of inflammatory cells and microfilaria were also seen in the spleen sections.

Molecular results

The sequencing of the *cox1* gene was used to characterize the five *Dirofilaria* isolates. A PCR was used to successfully produce the amplicons of 689 base pairs (bp) from all 5 specimens (one from each dog) (Figure 9). The comparison of the sequences with other available sequences in Genbank revealed that all five isolates were identical with each other and were all as *D. immitis* with 100% identity with sequences available in the NCBI database reported from other countries such as Iran (KT351849– KT351852), Australia (AJ537512), Hungary (KM452920, KM452921), Bangladesh (KC1078050), China (EU159111) and Italy (DQ358815).

Discussion

Canine dirofilariasis has been diagnosed worldwide. The global climatic warming and movement of people and other animals correlate with the dissemination of emerging infectious disease phenomena. Consequently, the movement of the microfilaremic infected dogs seems to be the essential factor contributing to the additional spreading of the *D. immitis*.

The prevalence and spreading of heartworm infestation in Iraq are underestimated (Alia et al., 2013). The disease was not reported and believed absent until 2009, when a high prevalence of infestation (72.72%) was reported, and researchers hypothesized that heartworm disease is usually present in the local and imported dogs in Iraq (Amall et al., 2009). Epidemiological studies have shown that dirofilariasis
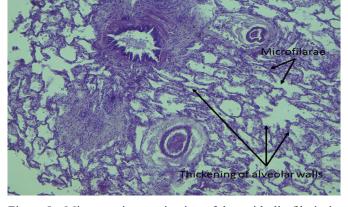

Figure 8 - Microscopic examination of dog with dirofilariasis. Lung section revealed thickening of alveolar walls and interstitial pneumonia, lymphoid cuffing of bronchi, bronchioles and vessels and fibrosis.
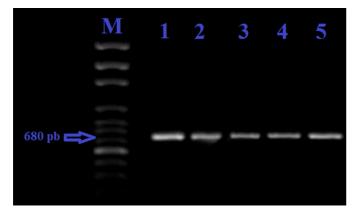

Figure 9 - Shows the five positive isolates amplified for *cox1* gene at 680 bp by PCR products, lane M: shows the molecular size marker.

is considered as an emergent parasitic disease of animals and humans (Simón et al., 2012).

The disease appeared to be more prevalent in the regions with temperate and tropical climates (Bolio-Gonzalez et al., 2007). Furthermore, *D. immitis* endemic circulation has been lately considered in most countries in the tropical, subtropical and temperate zone (Mccall et al., 2008b). Iraq is situated in the southwestern part of Asia and has favorable climatic condition with high temperature and moisture that encourages the distribution and development of the mosquito vector of *Dirofilaria*. Moreover, Iraq has a large number of stray dogs and wild carnivores that act as reservoir hosts of different parasites, including heartworm, and do not receive antiparasitic drugs (Al Salihi et al., 2017).

This study provides the first report on the occurrence and molecular characterization of *D. immitis* from imported (from different European countries and USA) working police dogs in Iraq. Dirofilariasis in working dogs was reported previously in southern France in a total of 207 military dogs and, five years later, in 180 dogs utilized by the army with high prevalence percentages, 46.8% and 37.2%, respectively (Chauve, 1997).

The result of blood parasitological survey of the current study revealed that 38.46% (15 of 39 examined police dogs) were diagnosed with moderate to severe microfilaremic *D. immitis*. This percentage indicates that the infestation in working dogs is high and compatible with results reported previously in Iraq (Amall et al., 2009), France (Chauve, 1997) Slovakia (Miterpáková et al., 2010) and South Korea (Song et al., 2003).

All these dogs were imported to Iraq and had their health certificate and travel passport; therefore, based on this information, these dogs might have become infested inside Iraq. Consequently, a possible scenario of infestation is that these dogs spent several weeks in different patrol tasks in the entrance of the cities. Moreover, *D. immitis* has colonized these areas because of the availability of many heartworm reservoirs (the stray dogs), suitable weather and high density of mosquitos as the intermediate vectors.

In this study, the infested dogs presented with classic clinical signs and clinical remarks of naturally occurring heartworm disease. All five dead dogs revealed respiratory distress including cough, dyspnea/ hyperpnea and syncope accompanied with weight loss, exercise tolerance weakness, fatigue, poor body condition and abdominal distension that might result from severe spontaneous thromboembolism after the natural death of many heartworms or due to treatment. These results are compatible with the previous ones (Venco et al., 2005; Kaewthamasorn et al., 2008),

according to which worms that die due to treatment cause the most severe damage, including thrombosis, granulomatous inflammation, corrugated and villous inflammation. Consequently, thromboembolism might cause acute decompensation by producing or provoking pulmonary hypertension, right heart failure, or pulmonary infarction.

Mccall et al. (2008a) assumed that both naturally dead adult heartworms and those dead due to treatment resulted in intensified inflammatory response due to the release of *Wolbachia* bacteria from dead and broken worms. The appearance of clinical signs of heartworm disease depends on the duration and severity of infestation that reveals the effects of the parasite on the pulmonary arteries, lungs and heart.

Both immature and mature heartworms are initially located in the caudal pulmonary vascular tree, then move to the principle pulmonary arteries, right heart and to the large veins, particularly in severe infestations. At this stage, the heartworms started their primary effect on the pulmonary arteries by the worm influences, including toxic substance, immunological response, and physical trauma accompanied by other pathological reactions (Mccall et al., 2008a).

The accurate clinical diagnosis and gross-histopathological investigations of *D. immitis* are the essential tools for development of a productive future treatment and prevention program for dogs. The results of this study showed that the gross pathology lesions were seen in all organs of the necropsied dogs; moreover, these lesions caused circulatory disorders made by severe vascular and cardiac parasitism and by the toxic effects of the parasites.

All gross lesions observed in this study, including the thrombus formation in the subcutaneous capillaries, the respiratory system lesions including the pulmonary arteries, and right ventricular dilatation were influenced by the presence of parasites in the ventricular cavity and the very soft and thin ventricular wall that was occupied with a large thrombus surrounded the parasites.

These results are compatible with gross pathological lesions described previously in reported cases (Stephen et al., 2017; Kaiser & Williams, 2004). Besides, the results of the hepatic, spleen and urinary tract lesions appeared in all cadavers.

These results are in agreement with previous researchers, who described the renal and myopathy lesions of *D. immitis* in natural infested dogs in Iraq (Enaam et al., 2011), membranous glomerulonephritis in dogs infested with *D. immitis* in Romania (Pasca et al., 2012), *D. immitis* as an

unusual clinical presence in Thailand (Kaewthamasorn et al., 2008), and the canine visceral leishmaniasis (vascular, hepatic and renal lesions) naturally infected by *Leishmania chagasi* in Rio de Janeiro, Brazil (Honse et al., 2013).

In the current study, the histopathological changes were observed in the respiratory system, particularly in the pulmonary arteries and lungs, such as endothelial damage, villous proliferation and progressive organization of the filariae in the pulmonary arteries. Moreover, a marked arterial wall disorder was present, due primarily to compression, and secondarily to an inflammatory process from early infiltration of plasma cell and lymphocyte, followed by fibrous organization and focal granuloma formation, in addition to the presence of the worms in the right pulmonary artery.

All these observations are compatible with previous studies on canine filariasis (Mccall et al., 2008a; Casey & Splitter, 1975; Atwell & Carlisle, 1982). The histopathological lesions found in the present study in the livers in all cases were compatible with previous observations reported by other researchers (Atwell et al., 1986). These changes, such as venous stasis and edema, occurred as consequences of the right heart failure (Mccall et al., 2008b).

In the current study, both glomerular and tubular histopathological lesions observed in kidneys are in agreement with the observations of canine membranous glomerulonephritis due to *D. immitis* infestation reported by other researchers (Mehlhorn, 2008; Pasca et al., 2012). The glomerular lesions due *D. immitis* were categorized as one of the immune complex types of membranous glomerulonephritis. Nevertheless, Casey & Splitter, (1975) mentioned that there was no recognized fundamental correlation with coexisting heartworm infestation and the glomerular lesions might occur from immune complexes in the blood due to the reaction between heartworm or microfilarial antigens.

However, they also mentioned that heartworm antigenic material might be liberated into the circulatory system, but most dogs infested with heartworms do not develop progressive renal lesions (Pasca et al., 2012). Moreover, the presence of immune-mediated glomerulonephritis in canine *D. immitis* is not unexpected as the lesions have been related with malaria (Mcgavin & Zachary, 2007) and *Babesia* infections (Ward & Kibukamusoke, 1969), which are also intravascular parasites.

Although some heartworm infestations in dogs are asymptomatic, nearly all infested dogs develop clinical signs due to their immune response to *D. immitis* resulting in diseases including glomerulonephritis and allergic pneumonitis. The secondary physical effects accompanied by the severe infestation in dogs can also lead to diseases such as congestive heart failure, caval syndrome, pulmonary hypertension and pulmonary thromboembolism (Annable & Ward, 1974).

In the current study, the observed severe clinical presentation, gross and histopathological changes of the dead dogs are compatible with the common pathophysiology of canine heartworm disease, in particular, the caval syndrome. Moreover, the live worms can also complicate the condition by arterial obstruction and vasoconstriction. Meanwhile, the dead worm produce thromboembolism (Frank & Heald, 2010).

According to Sironi et al. (1995), *D. immitis* harbors a special kind of gram-negative bacterium called *Wolbachia* that resides within the body of many filarial nematodes. It acts as an essential part in the pathogenesis steps and immune response to filarial infestation. This scientific fact would probably help in understanding the complex life cycle of filarial nematodes. The immunopathology of filarial disease is extremely complex, and the clinical manifestations of infestation rely on the type of immune response produced by the parasite.

This bacterium species is recognized to play a vital role in heartworm biology, reproduction, and long-term survival and the proinflammatory reaction to these bacteria might intensify the clinical picture perceived in heartworm-infested dogs and cats. Several researchers reported the real role of *Wolbachia spp* in the development of the inflammatory reaction associated with heartworm disease in dogs and cats (Bazzocchi et al., 2000; Bazzocchi et al., 2003: Morchon et al., 2004). Bazzocchi et al. (2000) found that in *Wolbachia* surface protein (WSP), the IgG immune response developed after *D. immitis* infestation in cats. Moreover, these antibodies against *Wolbachia spp*. increased after larvicidal therapy (Bazzocchi et al., 2000).

Likewise, in canines, WSP acts by activating neutrophils by prompting chemokinetic activity and IL-production (Kramer et al., 2005), supporting the observations that the presence of circulating antibodies against WSP in all dogs infested with *D. immitis* is independent of clinical of signs of heartworm disease (Morchon et al., 2004).

Positive immunohistochemical staining has also shown WSP in tissue samples (lung, liver, and kidneys) of deceased dogs with heartworm disease, and that *Wolbachia* organisms were localized within renal tubules and glomeruli and hepatic monocytes, besides appearing as extracellular bacteria in the lungs (Morchon et al., 2004). According to previous studies, *D. immitis* specimens were found in Iraq,

and the diagnosis was done according to morphological features of the parasite (Amall et al., 2009; Enaam et al., 2011; Alia et al., 2013). In the present study, the molecular characterization was done for *Dirofilaria* isolates from the necropsied dogs using PCR.

The *cox1* gene amplicons of 689 base pairs (bp) were successfully produced from all specimens and showed homology with sequences already deposited in NCBI database from other countries, such as Iran (KT351849– KT351852), Australia (AJ537512), Hungary (KM452920, KM452921), Bangladesh (KC1078050), China (EU159111), and Italy (DQ358815) (Venco, 2007; Bazzocchi et al., 2003).

Conclusion

In conclusion, a high prevalence of *D. immitis* was diagnosed in the imported police dogs and represents a significant veterinary problem. Moreover, the infestation most probably occurred inside Iraq, as these dogs were shown to be healthy by their health certificates before traveling inside the country. Furthermore, the result of this study showed that Iraq is one of the endemic areas for canine heartworm disease. The observed severe clinical signs and gross and histopathological changes revealed the typical occurrence of the disease in the dead dogs, and the severity of the thromboembolic lesions suggested that these dogs died due to the caval syndrome.

Additionally, PCR demonstrated that *D. immitis* is a species present in Iraq. The authors recommend further epidemiological studies on the occurrence of *D. immitis* in local dogs as well as imported dogs in all Iraqi governorates to better understand the epidemiological map of this disease and introduce both an active treatment and a preventive program.

Conflict of Interest

The authors state they have no conflicts of interest to declare.

Ethics

No ethical issues may arise after the publication of this manuscript.

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