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New possibilities in the diagnosis and treatment of canine heartworm disease

Ph.D. thesis

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List of abbreviations

Ag	antigen
AHS	American Heartworm Society
ALP	alkaline phosphatase
ALT	alanine transaminase
An.	*Angiostrongylus*
Ac.	*Acanthocheilonema*
BID	twice daily (bis in die)
CI	Blaker 95% confidence interval
CT	computer tomography
CTA	computer tomographic angiography
D.	*Dirofilaria*
DNA	deoxyribonucleic acid
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EOD	every other day
ELISA	enzyme-linked immunosorbent assay
ESCCAP	European Scientific Counsel Companion Animal Parasites
ESDA	European Society of Dirofilariosis and Angiostrongylosis
FDA	Food and Drug Administration
GABA	gamma-aminobutyric acid
GI	gastrointestinal
HW	heartworm
HWs	heartworms
HWD	heartworm disease
IgG	immunoglobulin G
im.	intramuscular
inj.	injection
L	larval stage
LMWH	low-molecular-weight-heparin
MDR-1	multi-drug resistance 1
mf	microfilaria/microfilariae
ML	macrocyclic lactone

1	MLs	macrocyclic lactones
2	PCR	polymerase chain reaction
3	PG	pressure gradient
4	PGP	P-glycoprotein
5	PHT	pulmonary hypertension
6	PTE	pulmonary thromboembolism
7	RCHF	right sided congestive heart failure
8	RPM	round per minute
9	rRNA	ribosomal ribonucleic acid
10	rWSP	recombinant *Wolbachia* surface protein
11	S.	*Spirocerca*
12	SAC	Small Animal Clinic
13	sc.	subcutaneously
14	SID	once daily (semel in die)
15	US	ultrasonography
16	UVMB	University of Veterinary Medicine Budapest
17	vmax	maximal velocity
18	WSP	*Wolbachia* surface protein

Summary

Heartworm disease (HWD), caused by *Dirofilaria immitis* is an emerging mosquito-borne disease in Carnivores, mainly in dogs, and could be found on most of the continents. HWD is an emerging disease also in Hungary, and several regions are already endemic, especially in the southern and eastern parts of our country. *Dirofilaria repens* causing mainly subcutan dirofilariosis also present in Asia and Africa, but not in the Americas. This nematode can also be found in several middle European countries including Hungary, mainly in similar regions as *D. immitis*. The concomitant occurrence of these *Dirofilaria* species has special importance, especially from differential diagnostic aspects, being their treatment also different. Proper diagnosis, therapy, and prevention of HWD is inevitable for protecting canine patients, and to inhibit potential human infections which might occasionally be transmitted by infected mosquitoes. In my thesis, I report on our research related to some new possibilities in the diagnosis and treatment of canine HWD.

In the first part of our research, we determined the possibilities of some serodiagnostic methods in heartworm (HW) infected dogs living in regions where concomitant infections of *D. immitis* and *D. repens* occur. The VetScan Ag test was used as a model for Ag tests and the PCR assay served as a standard for the parasitological diagnosis. Sensitivity and specificity were studied of this Ag test under these conditions. We also aimed to demonstrate if true occult dirofilariosis could be the explanation for positive Ag tests despite of a negative PCR result of *D. immitis.* In our cases with occult dirofilariosis, we have applied several, various antigen tests of different manufacturers for the diagnosis of HWD. These was based on the hypothesis that the chance for a cross-reaction (false positivity) among *D. immitis* and other helminths (including *D. repens*) is less when more than one HW Ag tests provide positive results from the same blood sample.

Altogether 71 dogs were included retrospectively into the study. These dogs were randomly selected from dogs arriving for HW screening examinations or with clinical suspect of HWD. The examination methods included the modified Knott test and in its positivity the polymerase chain reaction (PCR) methods to identify *D. immitis* and/or *D. repens* infections as well as a heartworm antigen (Ag) test (VetScan). By using PCR, 26 dogs were found positive only for *D. immitis* (Group 1), while 21 dogs for both, *D. immitis* and *D. repens* (Group 2). Group 3 included 24 dogs with *D. repens* infection only according to the PCR results. The sensitivity of the VetScan Ag test for the Group 1 and 2 animals proved to be 97.7% (95% Blaker confidence interval; CI 89.0% - 99.9%). The specificity of the VetScan Ag test, calculated from the results

of Group 3, was found to be 66.7% (95% CI 45.6% - 83.1%), which was lower than that reported from the USA, where *D. repens* does not occur. In cases when PCR results were positive for *D. repens* but negative for *D. immitis*, occult dirofilariosis was the likely explanation for the positive *D. immitis* Ag tests. These observations highlight the importance of performing more Ag tests simultaneously in those areas where both *Dirofilaria* species and occult dirofilariosis are present. This has special interest when a PCR cannot be performed in the lack of microfilariae in the peripheral blood or when this technique reveals only the presence of *D. repens*.

In the second part of my thesis, I present our scientific results related to the treatment of HWD. The standard therapy of HWD is recommended by the American Heartworm Society (AHS) which is effective and accepted internationally. During our research, we have modified and complemented the AHS protocol with some additional new medications and therapeutic measures to decrease the possible side-effects and to further improve the efficacy and outcome of the AHS therapeutic procedure. For these purposes, moxidectin was newly used as part of the complex therapeutic protocol. Probiotics were added to the application of doxycycline, in order to decrease the potential gastrointestinal side-effects of this antibacterial drug. The patients were sedated with butorphanol before the intramuscular application of the melarsomine injection into the paralumbar musculature. The exact place of the needle was determined with the help of ultrasonography, and the potential local complications were also followed with this imaging method.

The AHS complex therapeutic protocol modified and complemented as described above was used in 44 heartworm-positive canine patients. Microfilaremic dogs were pretreated with prednisolone and clopidogrel for one week before the first moxidectin application to avoid potential adverse (shock-like) reactions which could be caused by the dying microfilariae. Moxidectin was applied locally as spot-on solution, on the 1^{st}, 30^{th}, 60^{th}, and 90^{th} therapeutic days. On the 1^{st} day, dexamethasone and chloropyramine were used to avoid potential adverse effects mentioned above. During the 1^{st}-28^{th} days, doxycycline 10 mg/kg BID was given with probiotics. Adult heartworms were treated with melarsomine on the 60^{th}, 90^{th} and 91^{st} days. Butorphanol and dexamethasone were given just before melarsomine injections. The depth of the intramuscular injection site was determined by ultrasound examination of the lumbar muscles. From the 60^{th} day, dalteparin was applied for 10 days to decrease the chance of pulmonary thromboembolism, elicited by the destroyed adult heartworms.

Moxidectin did not cause adverse (shock-like) reactions, even in microfilaremic dogs. This might be also due to pretreating these dogs with prednisolone and clopidogrel before moxidectin application. By using moxidectin instead of ivermectin, a complete healing of each HWD patients was achieved and no death occurred even in severe (Clinical stage 3) cases, which results are remarkably better compared to previous studies regarding the outcome of therapy. The partial adulticide effect of moxidectin might have been added to that of melarsomine, which can be an explanation for the favorable (100%) recovery rate of our patients. Gastrointestinal side effects of doxycycline were observed only in 3 (6%) dogs, that healed after symptomatic therapy and by lowering the initial 10 mg/kg BID dose to 5 mg/kg BID. Transient anorexia and diarrhea were found in one (2%), as well as coughing and mild dyspnea in one (2%) animal as systemic post-therapeutic complications of melarsomine. These gastrointestinal side effects of doxycycline treatment were remarkably lower compared to previous studies, possibly due to probiotics supplementation. Butorphanol provided satisfactory sedation and analgesia during and after melarsomine injection. Ultrasonography was successfully applied to determine the location of the injection needle for melarsomine injection. This method allowed the follow-up of the course of local side effects which were milder compared to literary data. Namely, no local side effects were observed in 13 (30%) dogs, mild local side effects occurred in 29 (66%) patients, and severe local swelling only in 2 (4%) cases. All dogs recovered clinically by the 120th day and no microfilaraemia was seen that time by using the modified Knott test in 33/44 patients. A HW antigen test performed in 33/44 animals on the 271st day was also negative in all cases.

The third part of my thesis includes a case report on a dog with true occult dirofilariosis which caused severe clinical symptoms, that has not been published previously in Hungary. This case report is also an example of the diagnostic work-up and therapeutic management of severe HWD caused by occult dirofilariosis with special regard for applying several Ag tests in these cases. The latter has never been published as to our search through the international literature.

Összefoglalás

A kutyák szívférgessége, amelyet a *Dirofilaria immitis* idéz elő, egyre gyakoribbá váló, szúnyogok közvetítette betegség ragadozókban - különösen kutyákban - amely megtalálható a legtöbb kontinensen. Európában a szívférgesség korábban a mediterrán déli régiókban fordult elő, de manapság már terjed északi irányba is. Ez a tendencia elsősorban a globális felmelegedésnek tudható be, ami kedvező a szúnyog vektorok számára, de részben betudható a kutyákkal történő, gyakoribb nemzetközi utazásoknak is. Napjainkban a szívférgesség egyre gyakoribbá válik Magyarországon is, és számos régió már endémiásnak mondható, különösen hazánk déli és keleti területein. A *Dirofilaria repens* elsősorban a bőr alatti dirofilariosist idézi elő, és szintén jelen van Ázsiában, valamint Afrikában, azonban nem található meg az amerikai földrészen. Ez a féreg számos közép európai országban, köztük Magyarországon is jelen van, főképpen azokon a területeken, ahol a *D. immitis* is fellelhető. A két parazita együttes előfordulásának különleges jelentősége van, főképpen differenciál diagnosztikai szempontokból, mivel a gyógykezelésük is különböző. A szívférgesség megfelelő diagnosztikája, fertőzöttség esetén a terápiája, illetve a megelőzése nélkülözhetetlen ahhoz, hogy a kutyákat megvédjük a betegség következményeitől, és azért is, hogy meggátoljuk a potenciális humán fertőződéseket, amelyek alkalmanként előfordulhatnak a fertőzött szúnyogok közvetítése révén. Értekezésemben beszámolok néhány új lehetőségről a szívférgesség megállapításának és gyógykezelésének kapcsán végzett kutatómunkánk során.

Kutatásunk első részében olyan szívféreggel fertőzött kutyákban alkalmaztuk a parazitológiai diagnosztikai módszereket, amelyek olyan területekről származtak, ahol a *D. immitis* és a *D. repens* együttesen fordul elő. A kutatás indokát elsősorban az jelentette, hogy nem találtunk információkat a *D. immitis* antigéntesztek értékeléséről az ilyen régiókban élő kutyákra vonatkozóan. Modellként a VetScan antigéntesztet használtuk az antigéntesztek közül, és a PCR-eljárás szolgáltatta a parazitológiai diagnózis standardját. A szenzitivitás és a specificitás meghatározására ilyen feltételek mellett került sor az említett antigéntesztre vonatkozóan. Szintén szándékunkban állt annak tanulmányozása, miszerint a tényleges okkult dirofilariosis lehet-e a magyarázata a pozitív eredményt mutató szívférgesség antigénteszteknek azokban az esetekben, amikor a *D. immitis* PCR eredménye negatív volt, amelyet időnként a saját klinikai praxisunkban, korábban tapasztaltunk. Ilyenkor nem találhatók mikrofiláriák a perifériás vérben a szívférgesség során, a szervezet immunológiai védekező reakciójának következtében, és emiatt a kimutatásukra szolgáló Knott-teszt negatív eredményt ad. Az okkult szívférgesség eseteiben többféle, különböző gyártótól származó antigéntesztet vettünk igénybe a

szívférgesség diagnózisának céljából. Ezt arra a hipotézisünkre alapoztuk, miszerint a tévesen pozitív keresztreakciók esélye a *D. immitis* és más férgek (köztük a *D. repens*) között kisebb, amennyiben több antigénteszt is pozitív eredményt ad ugyanabból a vérmintából.

Összesen 71 kutyát vontunk be randomszerűen a kutatásba azok közül, amelyek a szívférgesség felderítésére szolgáló szűrővizsgálatokra vagy pedig a szívférgesség klinikai gyanújával érkeztek. A vizsgálati módszerek magukban foglalták a módosított Knott-tesztet, ennek pozitivitása esetén a PCR-vizsgálatot a *D. immitis* és/vagy a *D. repens* fertőzések azonosítására, valamint a fent említett szívféreg antigéntesztet. A PCR-eljárás során 26 kutya bizonyult csupán *D. immitis* pozitívnak (1-es csoport), míg 21 kutyában egyidejű *D. immitis* és *D. repens* fertőzést mutattunk ki (2-es csoport). A 3. csoport 24 olyan kutyát foglalt magában, amelyekben csak *D. repens* fertőzés fordult elő a PCR-eredmények alapján. A VetScan antigénteszt szenzitivitását az 1-es és a 2-es csoport adataiból határoztuk meg, és ez 97,7%-nak bizonyult (95%-os Blaker-féle konfidencia intervallum; KI 89,0% - 99,9%). A VetScan antigénteszt specificitását a 3-as csoport eredményeiből számoltuk ki, ami 66,7%-nak bizonyult (95% KI 45,6% - 3,1%), Ez az érték alacsonyabb azokhoz a tanulmányokhoz képest, amelyeket az Amerikai Egyesült Államokban végeztek, ahol a *D. repens* nem fordul elő. A pozitív *D. repens* és egyúttal negatív *D. immitis* PCR-eredmények esetén az okkult dirofilariosis volt a valószínű magyarázatunk a pozitív *D. immitis* antigénteszteket illetően. Ezek a megfigyelések megvilágítják a jelentőségét annak, hogy egyidejűleg célszerű több antigéntesztet igénybe venni azokban a régiókban, ahol mindkét *Dirofilaria*-faj jelen van, és az okkult dirofilariosis egyaránt előfordul. Ennek különös jelentősége van, ha a PCR-vizsgálat nem végezhető el a microfilaraemia hiányában, vagy pedig csak a *D. repens* jelenléte mutatható ki.

Az értekezésem második részében bemutatom azokat az új tudományos eredményeinket, amelyek a szívférgesség gyógykezeléséhez kapcsolódnak. A szívférgesség standard terápiáját az Amerikai Szívférgesség Társaság (American Heartworm Society, AHS) ajánlása alapján végzik világszerte, amely hatékony módszernek mondható. Ez az összetett terápiás protokoll magában foglal egy makrociklikus laktont (napjainkig az ivermektint) a mikrofiláriák, valamint az L3 és az L4 lárvák elpusztítása céljából, a doxiciklint a szimbionta *Wolbachia* baktériumokkal szemben és az adulticid hatású melarzomin-dihidrokloridot. Ugyanakkor mellékhatások és komplikációk egyaránt előfordulhatnak ezeknek a gyógyszereknek a használatakor. A kutatásunk során módosítottuk és kiegészítettük az AHS protokollt néhány további, ebből a szempontból újonnan alkalmazott gyógykezelési eljárással, annak érdekében, hogy csökkentsük a mellékhatásokat, és tovább fokozzuk a terápia hatékonyságát, illetve

javítsuk a betegség kimenetelét. Ebből a célból, makrociklikus laktonként a moxidektint használtuk az ivermektin helyett, tudomásunk szerint elsőként a szakirodalomban, ami a moxidektin alkalmazását illeti, a szívférgesség komplex terápiájának részeként. A doxiciklin kezelés mellé probiotikumokat adtunk annak érdekében, hogy csökkentsük e gyógyszer potenciális gastrointestinalis mellékhatásait. Pácienseinket butorfanollal bódítottuk a melarzomin injekció applikációja előtt. Az injekciós tű pontos helyét ultrahangvizsgálat segítségével állapítottuk meg paralumbalis izmokban, és ezzel a módszerrel követtük nyomon a potenciális helyi szövődményeket is.

Az AHS komplex terápiás protokoll fent leírtaknak megfelelően módosított és kiegészített változatát alkalmaztuk 44 szívféreg-pozitív kutyában. A mikrofiláriás pácienseknek előzetesen prednizolont és klopidogrélt adtunk egy hétig, az első moxidektin kezelés előtt, a potenciális adverz (sokkszerű) reakciók megelőzésére, ami a mikrofiláriák pusztulásakor fordulhat elő. A moxidektint az 1., a 30., a 60. és a 90. terápiás napon, helyileg applikáltuk, rácseppentő oldat formájában. Az első napon dexametazont és kloropiramint kaptak azok a kutyák, amelyek perifériás vérében mikrofiláriák voltak kimutathatók, annak érdekében, hogy elkerülhessük a potenciális adverz (sokkszerű) reakciókat. Az első 28 napban doxiciklint alkalmaztunk 10 mg/kg/12h dózisban, probiotikum kiegészítés mellett. A felnőtt szívférgek ellen melarzomint injektáltunk a 60., 90 és a 91. napokon. A butorfanolt és a dexametazont közvetlenül a melarzomin injekció előtt adtuk be a microfilaraemiás kutyáknak, a fent említett adverz reakciók elhárítására. Az intramuscularis injekciók helyét és mélységét ultrahangvizsgálattal határoztuk meg a paralumbalis izomzatban. A 60. naptól dalteparint adtunk 10 napig, hogy csökkentsük a pusztuló adult szívférgek okozta thromboembolia veszélyét a tüdőben.

A moxidektin nem idézett elő adverz reakciókat még a mikrofiláriás kutyákban sem. Ehhez hozzájárulhatott az is, hogy az ilyen pácienseinket prednizolonnal és klopidogréllel előkezeltük a moxidektin applikációja előtt. A moxidektinnek az ivermektin helyetti alkalmazása során teljes gyógyulást tudtunk elérni valamennyi szívférges kutyában, és nem fordult elő elhullás, még a súlyos (3. klinikai) stádiumba sorolt pácienseknél sem. Ezek az eredmények kedvezőbbek az eddigi tanulmányokban közöltekhez képest, a terápia kimenetelét illetően. A moxidektin részleges adulticid jellege is hozzájárulhatott a melarzomin hatásához, ami az egyik magyarázata lehet pácienseink kedvező (100%-os arányú) gyógyulásának. A doxiciklin gastrointestinalis mellékhatásait csupán 3 kutyában (6%) figyeltük meg, amelyek a tüneti kezelés után és a kezdeti 10 mg/kg/12h adag 5 mg/kg/12h csökkentésére gyógyultak. Átmeneti étvágytalanság és hasmenés fordult elő egy (2%) kutyában, valamint köhögés és enyhe

nehezített légzés egy másik (2%) kutyában, mint a melarzomin szisztémás posztterápiás komplikációja. A doxiciklin gastrointestinalis mellékhatásai lényegesen ritkábban fordultak elő, és enyhébbek voltak a korábbi tanulmányokhoz képest, feltehetően a probiotikumokkal való kiegészítésnek tulajdoníthatóan. A butorfanol megfelelő bódítást és fájdalomcsillapítást biztosított a melarzomin injekció beadásakor és azt követően. Az ultrahangvizsgálatot sikeresen alkalmaztuk az injekciós tű helyének meghatározására a melarzomin injekció applikációja során. Ez a módszer lehetővé tette a helyi mellékhatások nyomon követését is, amelyek enyhébbek voltak a szakirodalmi adatokhoz képest. Eszerint nem volt mellékhatás 13 (30%) kutyában, enyhe helyi mellékhatások mutatkoztak 29 (66%) páciensben és súlyos helyi duzzanat mindössze 2 (4%) esetben alakult ki. Valamennyi kutya klinikailag gyógyult volt a 120. napon, és nem voltak mikrofiláriák kimutathatók ebben az időben a módosított Knott-teszttel vizsgált 33/44 páciensünkben. Szívférgesség antigéntesztre 33 páciensünkben került sor az összesen 44 kutyából a 271. napon, amely valamennyi esetben negatívnak bizonyult.

Az értekezésem harmadik része egy esetismertetést foglal magában egy valódi okkult dirofilariosis okozta, súlyos betegségben szenvedő kutyáról. Az elsődleges célja ennek az esetismertetésnek az volt, hogy demonstráljuk a fő klinikai tüneteket, valamint a diagnosztikai és terápiás módszerek lehetőségeit a valódi okkult dirofilariosis során, amelyről korábban még nem publikáltak Magyarországon. Ez az esetismertetés egyúttal példát szolgáltat a diagnosztikai és gyógykezelési tevékenység lehetőségeire az okkult dirofilariosis által okozott súlyos szívférgesség eseteiben, különös tekintettel több antigénteszt egyidejű alkalmazhatóságára. Tudomásunk szerint ez utóbbiról még nem jelent meg közlemény, a nemzetközi szakirodalom áttekintése alapján.

Introduction and aims of the study

Etiology and occurrence of the disease

Heartworm disease (HWD), caused by *Dirofilaria immitis* is an emerging mosquito-borne disease in carnivores, mainly in dogs, and could be found on most of the continents (McCall et al. 2008, Morchón et al. 2012a, Simón et al. 2012, Morchón et al. 2022). This vector-borne species belongs to the Nematoda phylum, Spirurida order, Onchocercidae family, *Dirofilaria* genus and subgenus. The main host of *D. immitis* is the dog, however it can be found in other carnivores as well e.g., cats, ferrets, wild carnivores, and red pandas (van Zeeland and Schoemaker 2022). The disease also has zoonotic properties, but it is abortive in the human host, therefore no mature parasites develop, or microfilariae occur (Pampiglione et al. 1995, Kassai 2003, 2011). The larvae in people migrate into the lung, dying there and causing benign nodular lesions, only with mild clinical signs. These alterations have differential diagnostic properties during radiologic and/or CT-examinations (Morchón et al. 2012a, Simón et al. 2012, Malik et al. 2016). The spreading of canine HWD could also predict that the number of human cases will also increase in endemic regions (Laidoudi et al. 2021, Mendoza-Roldan et al. 2021, Miterpáková et al. 2022).

In Europe, HWD was formerly found at the tropical and in the south Mediterranean regions e.g., Spain, Portugal, Greece, France, and Turkey. Its spreading towards the North is due to global warming being favorable for the mosquito vectors (ESDA Guideline 2017, Drake and Wiseman 2018, ESCCAP Guideline 05. 2019, Farkas et al. 2020, Fuehrer et al. 2017) and to the increase in international travelling with dogs, HWD appeared in middle and Eastern Europe e.g., Serbia, Croatia, Czech Republic, Slovakia, and Romania. The unrecognition of the disease can also be a reason of its spreading (Genchi et al. 2005, Morchón et al. 2012a, Simón et al. 2012, Capelli et al. 2018, Farkas et al. 2020, Fuehrer et al. 2021, Morchón et al. 2022). In Hungary, wild carnivores i.e., red foxes and golden jackals might also play a role in the transmission of HWD (Tolnai et al. 2014).

In Hungary, the first occurrence of HWD was diagnosed in imported dogs, and their necropsy findings were published (Boros et al. 1982). Vörös et al. (2000) reported on an imported dog infected with *D. immitis*, describing the clinical signs and pathology of the disease. The first autochthonous case was diagnosed in 2007 and described by Jacsó et al. (2009). In the following years, increasing number of indigenous cases occurred in the country and HWD became

endemic in Southern and Eastern Hungary (Farkas et al. 2014, Bacsadi et al. 2016a, Bacsadi et al. 2016b, Trájer et al. 2016, Farkas et al. 2020).

Other nematodes being important in the differential diagnosis of heartworm disease are *D. repens* (Onchocercidae family, *Dirofilaria* genus, *Nochtiella* subgenus), rarely *Spirocerca lupi* (Spirucercidae family), *Angiostrongylus vasorum* (Rhabditida order, Angiostrongylidae family), and *Acanthocheilonema reconditum* (Onchocercidae family, *Acanthocheilonema* genus) (Manfredi 2001, Kassai 2003, 2011). Occurrence of *An. vasorum* has been reported in dogs several times in Hungary (Majoros et al. 2010, Csöndes et al. 2015, Schnyder et al. 2012, Nemes et al. 2016). There are publications on *Spirocerca lupi* from our country as well (Bokori et al. 1956, Széll et al. 2001, Psáder et al. 2017).

D. repens causing mainly subcutan dirofilariosis also present in Asia and Africa, but not in the Americas (Simon et al. 2012, Jacsó 2014, Genchi and Kramer 2017, Capelli et al. 2018). *D. repens* is known in Hungary, and in several middle European countries (Fok et al. 1998, Széll et al. 1999, Jacsó and Fok 2006, Spasojevic Kosic et al. 2012, Jacsó 2014, Cabanová et al. 2015, Härtwig et al. 2015, Ciuca et al. 2016a, Ciuca et al. 2016b, Fuehrer et al. 2016, Farkas et al. 2020). In Hungary, the first autochthonous case of was published at the end of the 1990's (Fok et al. 1998). However, the occurrence of the disease was already suspected from 1950 (Kotlán 1951). Clinical appearence, as well as potential therapeutic and preventive methods regarding *D. repens* infections have been published by the European Society of Dirofiariosis and Angiostrongylosis (ESDA) (ESDA Guideline 2017).

Life cycle of D. immitis

The life cycle of *Dirofilaria immitis* is indirect, which takes about 6-9 months (Hoch and Strickland 2008a, Bowman and Atkins 2009, Farkas and Vörös 2015, ESDA Guideline 2021, ESCCAP Guideline 05 2019). The matured female releases microfilaria (mf) into the host's blood stream of the infected dog and the mf must enter a suitable female mosquito during blood sucking (Manfredi et al. 2007, Nelson et al. 2020). The main vector species in Hungary are *Aedes vexans*, *Anopheles maculipennis* and *Culex pipiens* (Jacsó 2014). Other mosquito species can also be vectors for the disease (Nelson et al. 2020). In the mosquito, the microfilaria develops into a first-stage larva (L1) and after 2 molts becomes the infective third-stage larva (L3) (Hoch and Strickland 2008a, Bowman and Atkins 2009). The temperature of the environment greatly influences the length of its development. The fastest development takes

about 10-14 days at 27 °C. Below 14 °C, the transformation stops, however, if the temperature will rise, the larva develops further (Bowman and Atkins 2009, Nelson et al. 2020). The fully grown L3 will migrate to the labium of the mosquito from where - during the blood-sucking of the vector – it will get in a drop of hemolymph on the skin of the host and migrate into the subcutaneous tissue via the puncture of the infected mosquito (Manfredi et al. 2007, Nelson et al. 2018). It is interesting, that there is a natural inhibiting factor to decrease the number of infective larvae in the vector. The immune system of the mosquito recognizes the developing larvae as an antigen, and can injure the cuticle of the mf, therefore it cannot molt to L3 (Chandrasekharan et al. 1994, Manfredi et al. 2007, Simón et al. 2012).

The 3rd molt begins on day 3 in the host. By day 21, most of fourth larvae (L4) have migrated to the abdomen of the dog, and by day 41, they may be recovered from either the abdomen or the thorax. L3 and L4 migrate between muscle fibers. The last molt into juvenile adults occurs between days 50 and 70 after infection. The juvenile worm (L5) reaches the smallest pulmonary arteries as early as day 67 after the infection of the dog. The juvenile worms grow and get into the greater arteries (Nelson et al. 2018). The 2-3 cm long worms reach their final length at the 6-7th months. The length and the width of the maturated female is 25-30 cm and 1-1.3 mm, respectively. The adult male is 12-20 cm long, its width is 0.7-0.9 mm. Sexual maturity occurs about day 120 post infection, patent infections (i.e., having circulating microfilariae) as early as 6 months but usually by 7 to 9 months after infection (Manfredi et al. 2007, Nelson et al. 2014, 2020). The lifespan of the adult worms and the microfilariae is about 5-7 and 1-2 years, respectively.

The endosymbiotic *Wolbachia* bacteria e.g., *Wolbachia pipientis*, have important roles in living together with all stages of heartworms. These bacteria support the development and reproduction of *D. immitis*. The bacterial endotoxin produced by *Wolbachia* bacteria are implicated in the pathogenesis and immunological response to infection (Kozek 2005, Kramer et al. 2008, Kramer and Genchi 2014, Nelson et al. 2017).

Pathogenesis of HWD

The major consequences of HWD arise within the lungs (Hoch and Strickland 2008a, Bowman and Atkins 2009, Farkas and Vörös 2015, Vörös 2019). The adult worms induce reactive endothelial proliferation within the pulmonary arteries by decreasing the elasticity and diameter of their wall. Villous myointimal proliferation also occur. Later, edema formation and

perivascular (inflammatory) tissue reactions happen within the parenchymal parts of the lungs, and even fibrosis as well as partial lung consolidation. Vasoconstriction of the pulmonary arteries, secondary hypoxemia, endothelin-1 release as well as detrimental compounds released by the HWs contribute to the pathophysiology of HWD. As a consequence of this pathophysiology, pulmonary vascular resistance will increase, resulting in pulmonary hypertension (PHT). Spontaneously and/or during the treatment dying and fragmented HWs cause further damage of the pulmonary arteries together with thrombus formation. These fragments and thrombi can elicit embolization and occlusion of the vessels. Exercise of the patients increase pulmonary blood flow pressing the died and collapsed worms, their fragments, and the thrombi even into the smaller pulmonary arteries (Rawlings et al. 1983, Bowman and Atkins 2009, Nelson et al. 2020).

The damaged pulmonary arteries will be tortuous and dilated, mainly because of the increased vascular resistance and blood perfusion. Pulmonary alterations, especially PHT will cause chronic right heart pressure overload of the right heart, resulting in concentric myocardial hypertrophy with right ventricular dilation as well as increased diastolic ventricular pressure with secondary tricuspid and pulmonary valvular insufficiency. Right-sided congestive heart failure will be the sequel in severe cases (Ames and Atkins 2020, Nelson et al. 2020, Ware and Ward 2020). If there are several adult HWs within the lungs, they can wander to the main pulmonary artery and even to the right heart. The latter situation can result in the so-called caval syndrome especially in small dogs. This is the most severe clinical form of HWD due to the occlusion of the lumen of the right heart and even the entrance of the caval veins (Yoon et al. 2013, Chickweto et al. 2014).

Besides the cardiorespiratory system, other organs can also be damaged e.g., the liver and the kidneys (Niwetpathomwat et al. 2007). In the pathophysiology of renal alterations, not only the *D. immitis*, but the symbiotic *Wolbachia* bacteria take a role as well. They contain *Wolbachia* surface protein (WSP), causing an immunoreaction and damaging the lungs as well as the kidneys (Kramer et al. 2008, Morchón et al. 2012b, Nelson et al. 2014). *D. immitis* and also *D. repens* can cause glomerular injury and proteinuria (Morchón et al. 2012b, Carretón et al. 2013, Falus et al. 2022). Amyloidosis, glomerulosclerosis, immune mediated glomerulopathy and chronic interstitial nephritis can also occur (Paes-de-Almeida et al. 2003, Preyß-Jägeler et al. 2022). Renal thrombosis can develop, if the microfilaria burden is high and they block the renal capillaries as well (Carreton et al. 2013).

Clinical signs and classification

The beginning of the disease is usually symptomless, even for 1-2 years. However, if the HWD is left untreated, the clinical consequences could be severe and even death can/will occur. The severity of HWD depends on the following factors (Nelson et al. 2014, 2018, Vörös 2019, Ware and Ward 2020):

1. The number of juvenile and adult heartworms. If more worms are in the animal, the disease is usually more severe.
2. The length of the infection. Longer infection might cause more severe symptoms.
3. Interaction between the host and the parasite. Strong immune response can cause severe clinical signs, despite the low worm burden.
4. Physical burden. Increased physical activity (e.g., in working dogs) can elicit severe symptoms due to the pathologic changes in the lungs.
5. Size of the animal. In smaller dogs, similar worm burden can cause more severe clinical signs than in larger ones.
6. The pathological effects of *Wolbachia* bacteria (see their role above).

The severity of HWD can be classified as to the results of the physical examination as well as to the radiological findings as listed in **Table 1** (Farkas and Vörös 2015, Ware and Ward 2020).

In Stage 1, the dogs do not show clinical signs, or only mild coughing occurs. By the time going, the clinical signs are worsening. In Stage 2, exercise intolerance, coughing is present, and abnormal lung sounds can be heard. In stage 3, these symptoms worsen, and signs of right-sided heart failure (ascites, hepatomegaly) also develop. The most severe Stage 4 is the so called caval syndrome. Its most frequent clinical signs are quick worsening, severe lethargy, as well as hemoglobinemia and hemoglobinuria (Di Sacco and Vezzoni 1992, Farkas and Vörös 2015, Nelson et al. 2020). The radiological alterations affect mainly the pulmonary arteries and – especially later – the interstitium of the lungs as listed in **Table 1**.

Stage		Symptoms	Radiographic changes
1.	Mild	Asymptomatic or mild coughing	None
2.	Moderate	Coughing, fatigue, abnormal breathing sounds	Moderate enlargement of the pulmonary arteries; ± perivascular and mixed alveolar/interstitial infiltration, possibly right heart enlargement
3.	Severe	Coughing, fatigue, dyspnea, abnormal cardiac and respiratory sounds (cardiac murmurs), enlarged liver (hepatomegaly), Adams-Stokes syndrome (syncope due to hypoxemia), ascites, death	Moderate or severe enlargement of pulmonary arteries, abnormal, torturous shape, and course; perivascular or diffuse, mixed alveolar/ interstitial infiltration; enlargement of the right heart; ± signs of thromboembolism: worsening of the pulmonary infiltration, mainly as an alveolar pattern, in the caudal lobes
4.	Caval syndrome	Sudden worsening (severe lethargy and weakness), hemoglobinemia, hemoglobinuria	More severe forms of the aforementioned changes, mainly the enlargement of the right heart and the pulmonary trunk (main pulmonary arteries)

Table 1. Stages of heartworm disease based on the clinical symptoms and radiologic findings (*Farkas and Vörös 2015, Ware and Ward 2020*)

Diagnosis of heartworm disease

The relevant parasitological methods have their distinguished role in the diagnosis of HWD (Hoch and Strickland 2008a, Bowman and Atkins 2009, Farkas and Vörös 2015, ESDA Guideline 2017).

For detecting the mf, the modified Knott test is used mostly (Jacsó and Fok 2006, Majoros and Juhász 2015a, Majoros and Juhász 2015b, Genchi et al. 2018). The Knott technique is rather applicable for detecting microfilaraemia without definitive distinction of *D. immitis* and *D. repens* regarding the everyday clinical practice (Magnis et al. 2013).

The most sensitive and reliable diagnostic technique for in vivo diagnosis of blood filarioids is the polymerase chain reaction (PCR) technique as a molecular biological method (Favia et al. 1996, Rishniw et al. 2006, Latrofa et al. 2012, Rojas et al. 2015). This method allows definitive determination and distinction of *D. immitis* and *D. repens* if there are at least 4 mf in 1 mL peripheral blood (Gioia et al. 2010).

The circulating HW antigens are produced only by the adult female *D. immitis* worms (Roth et al. 1993, Goodwin 1998, Atkins 2003). Some factors e.g., the number of adult female worms, or the amount of the circulating antigens affect the sensitivity and specificity of the serological methods (Roth et al. 1993, Lee et al. 2011, Aron et al. 2012, Burton et al. 2020). Antigen (Ag) tests are especially important in cases of occult dirofilariosis (more precisely occult heartworm infection) when no mf can be detected in the peripheral blood despite a persisting HW infection with the presence of adult female worms. Occult dirofilariosis can have various etiologies e.g., previous macrocyclic lactone application for prevention, unisex infections, or when the mf are eliminated from the peripheral blood by the immune system of the infected dog. The latter phenomenon is called true occult dirofilariosis (Wong et al. 1973, Rawlings et al. 1982, Bagi et al. 2017). The Knott test and the PCR assay is not indicated for in vivo determination of occult dirofilariosis, as there is no circulating mf. Therefore, the diagnostic accuracy of the Ag tests is of utmost importance in occult dirofilariosis, as the sole laboratory diagnostic method in these cases (Nelson et al. 2020). This is especially true in those regions, where both *D. immitis* and *D. repens* may occur either separately or simultaneously in infected dogs (Ionica et al. 2015, Ciuca et al. 2016a, b, Bagi et al. 2017, Farkas et al. 2020).

Further clinical diagnostic methods

Diagnostic imaging techniques are applied mainly in HWD to determine the severity of the disease which strongly influences the complexity of the treatment and outcome of the disease.

Respiratory radiology can help to show the pulmonary changes caused by HWD and to diagnose or rule out other pulmonary pathologies. The radiographs should be done in (possibly right and left) lateral as well as in dorsoventral projections, for visualizing the lungs including their pulmonary vasculature. The width of the pulmonary arteries should not be larger than the width of the ninth rib or the diameter of the relevant bronchus (Ware 2014). At the early phase of the disease, mostly the smaller arteries are affected especially at the dorsocaudal edges of the lung lobes. In progressed HWD, the main pulmonary arteries as well as the larger branches are dilatated, torturous and truncated (Thrall and Calvert 1983, Losonsky et al. 1983, Atkins 2010). In addition, an interstitioalveolar pattern of the lungs is also visible as shown on **Figure 1** and **Figure 2**. In severe cases, cardiomegaly can also be diagnosed, mainly as the enlargement of the right heart (Rawlings 1986, Calvert and Rawlings 1988, Bowman and Atkins 2009, Nelson et al. 2020).

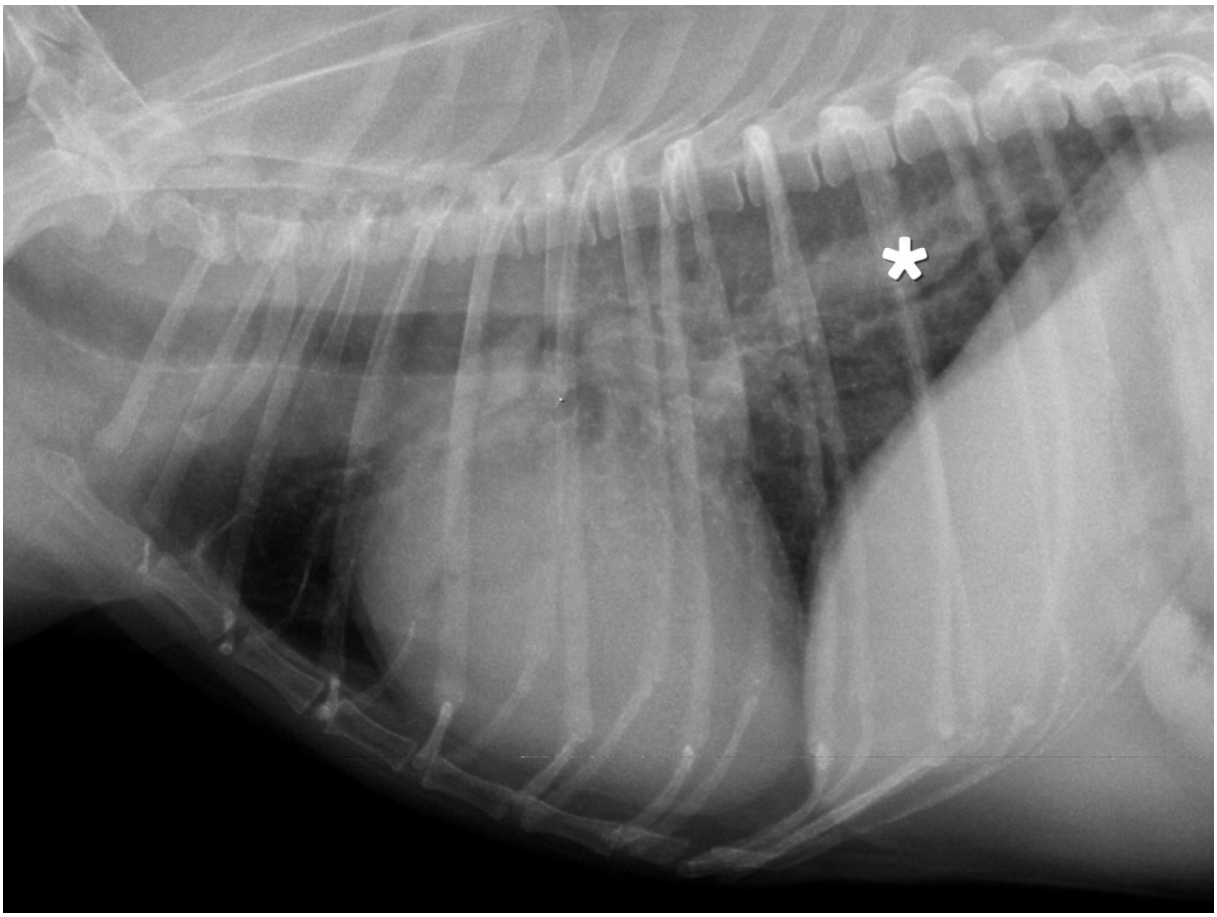


Figure 1. Lateral radiograph of the thorax of a dog with severe (Clinical Stage 3) heartworm disease. The lobar arteries are dilated and tortuous, particularly in the caudal lung lobes (*). Some patchy and diffuse interstitial patterns also appear in the caudal lung lobes. From Becker et al. 2022b. (With permission.)

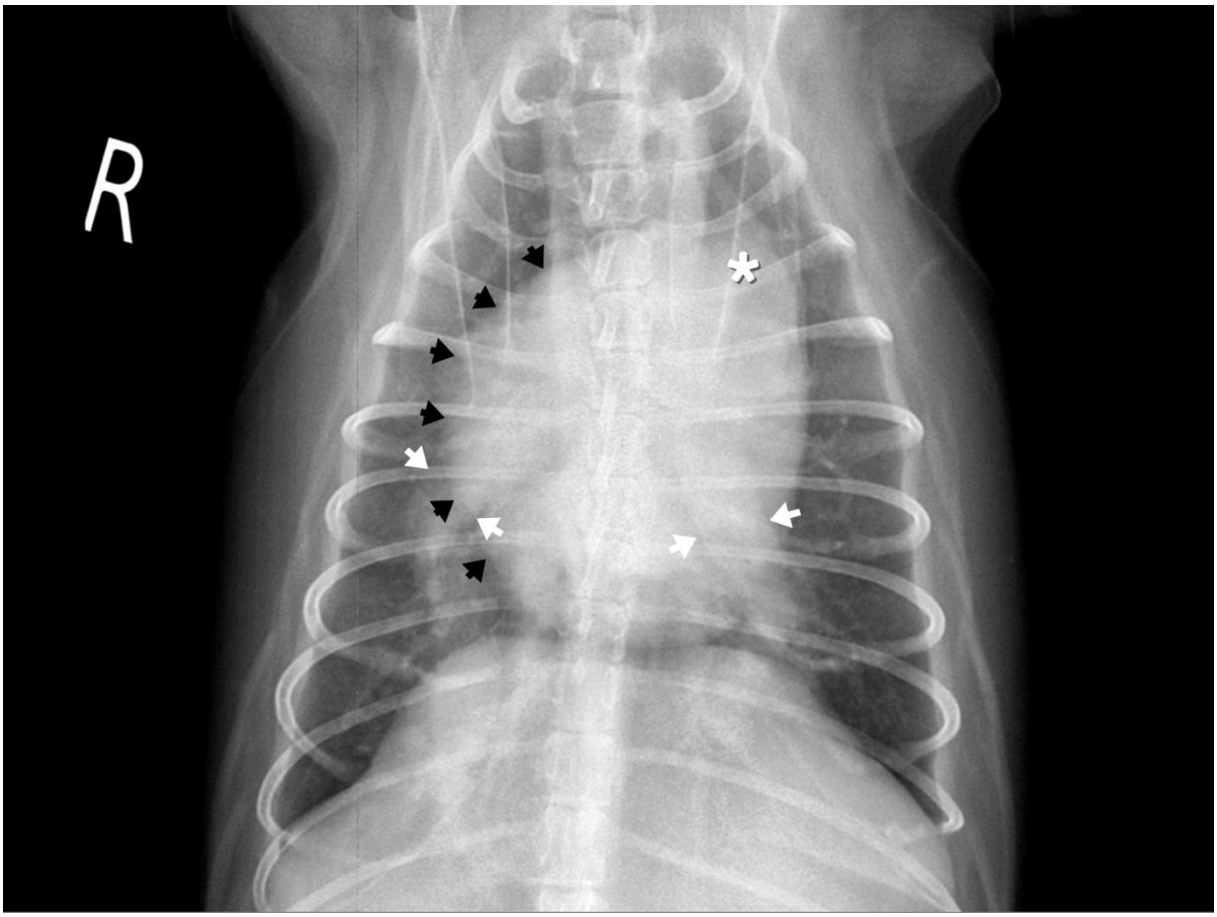


Figure 2. Ventrodorsal radiograph of the dog seen in Figure 1. Severe (Clinical stage 3) heartworm disease. The caudal lobar arteries are dilated (white arrows). The cardiac shadow has a reverse D shape because of the dilated right heart (black arrows). The bulging of the cardiac silhouette in the left craniolateral segment may indicate dilatation of the pulmonary trunk (*). From Becker et al. 2022b. (With permission.)

Echocardiography reveals the cardiovascular changes elicited by the elevation of the pulmonary arterial pressure e.g., dilation and hypertrophy of the right ventricle mainly in advanced cases. Right atrial dilatation and secondary tricuspid or pulmonary insufficiency can also occur. These pathologies contribute to assess the severity of HWD. Measuring the grade of tricuspid insufficiency is useful to quantify pulmonary hypertension as demonstrated on **Figure 3**. In mild cases (Clinical stages 1 and 2) echocardiography is an option being beneficial to follow up the cardiovascular changes during the therapy. In Stage 3, it is inevitable to perform, because of the high prevalence of pulmonary hypertension and right-sided heart failure, both needing appropriate therapy.

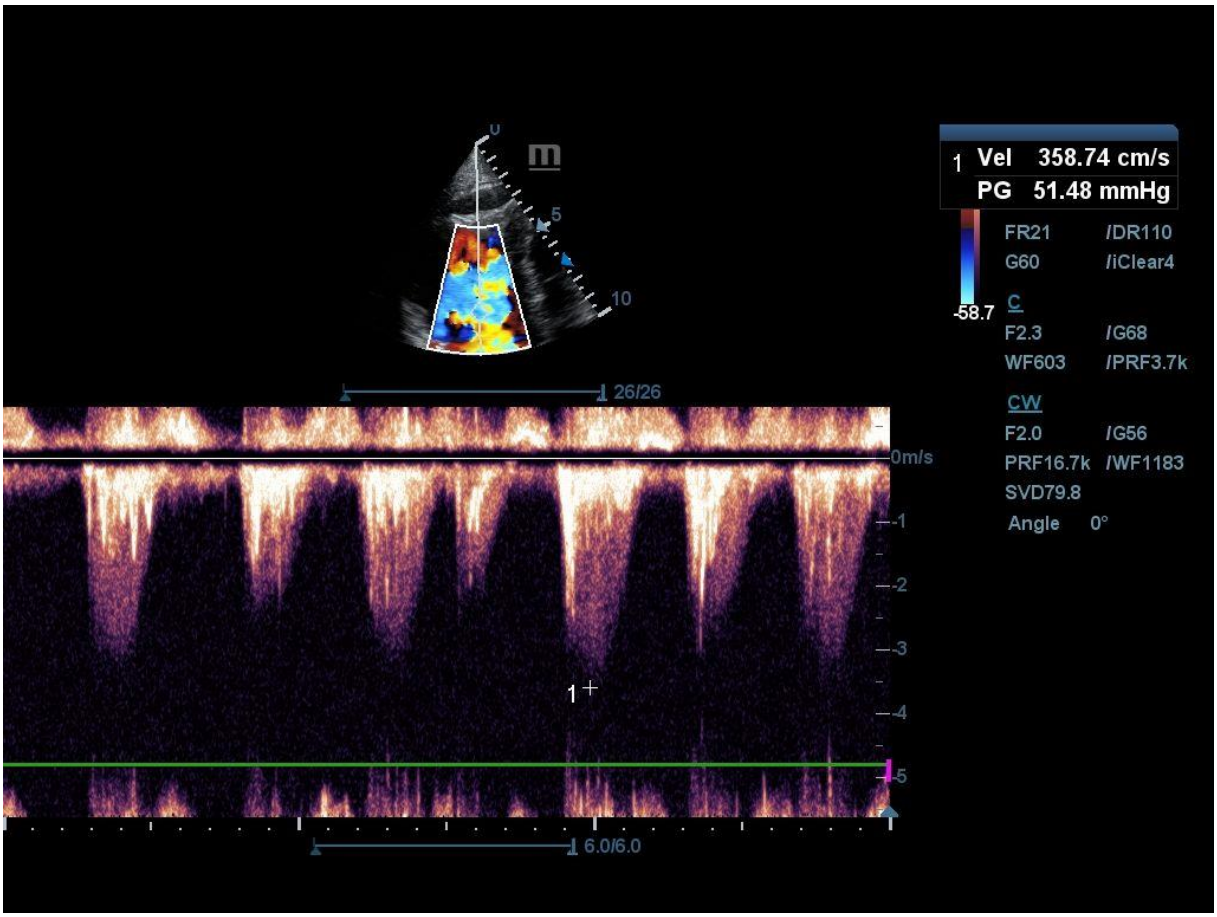


Figure 3. Severe (Clinical stage 3) heartworm disease in a dog. The maximal speed (Vel) of the tricuspid regurgitation is 358.74 cm/sec whilst the calculated pressure gradient (PG) yields 51.48 mmHg, representing tricuspid insufficiency caused by pulmonary hypertension (PHT). Continuous Doppler echocardiographic image, left apical, four-chamber view. On the top, the color Doppler part of the duplex echocardiographic image can be seen with the color signal of obvious regurgitation. Left apical, caudal, four-chamber view. From Becker et al. 2022b. (With permission.)

In some cases (mainly in Clinical stage 2 and 3), adult heartworms are visible in the main pulmonary artery **(Figure 4).**

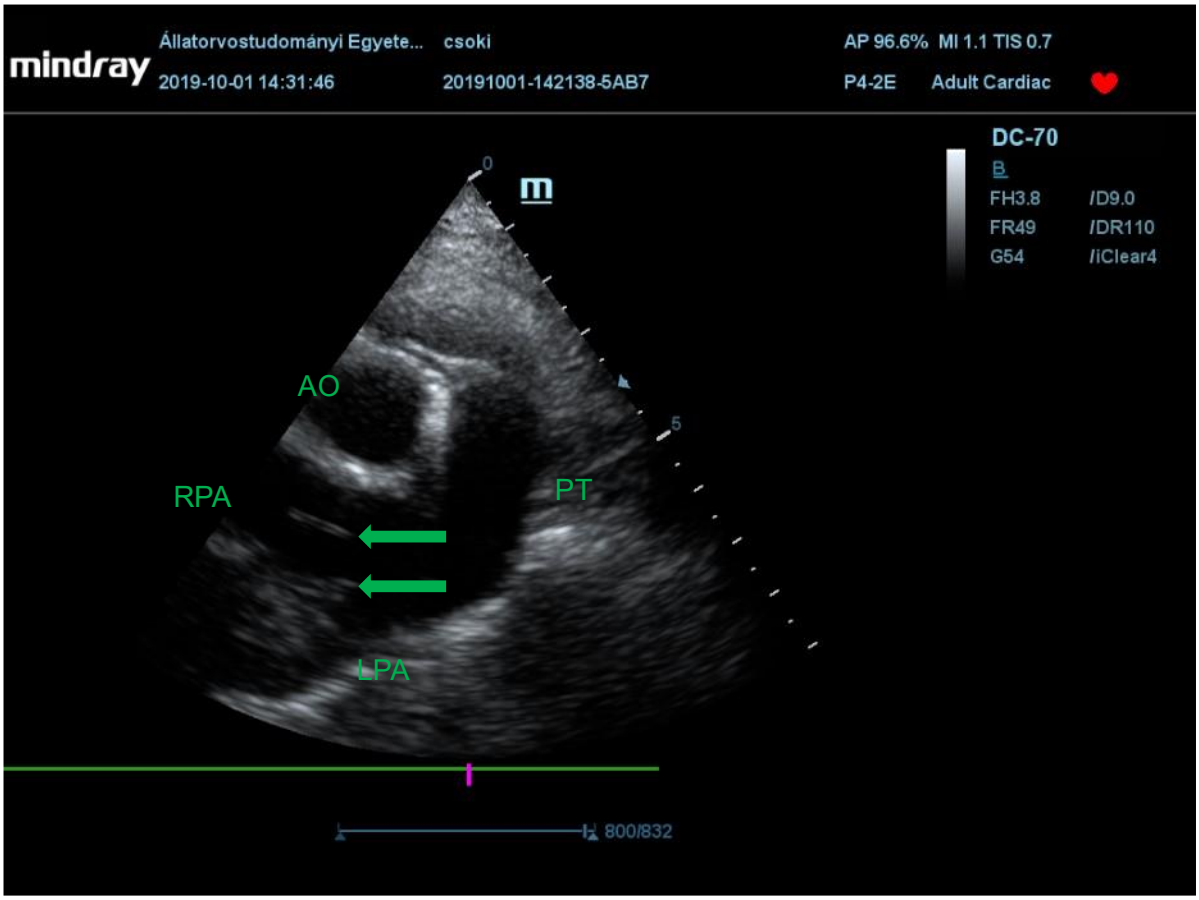


Figure 4. Cross-sections of adult heartworms (arrows) in the right pulmonary artery of a dog (Clinical stage 2). Two-dimensional echocardiographic image. Right parasternal short axis plane at the bifurcation of the main pulmonary artery. AO: aorta, PT: pulmonary trunk, RPA: right pulmonary artery), LPA: left pulmonary artery. (Courtesy of Dr. Károly Vörös.)

In severe HWD, the adult worm can also be present in the right atrium as well as in the orifice of the tricuspid valve especially if caval syndrome develops (Stage 4). This syndrome can only be diagnosed by echocardiography **(Figure 5).**

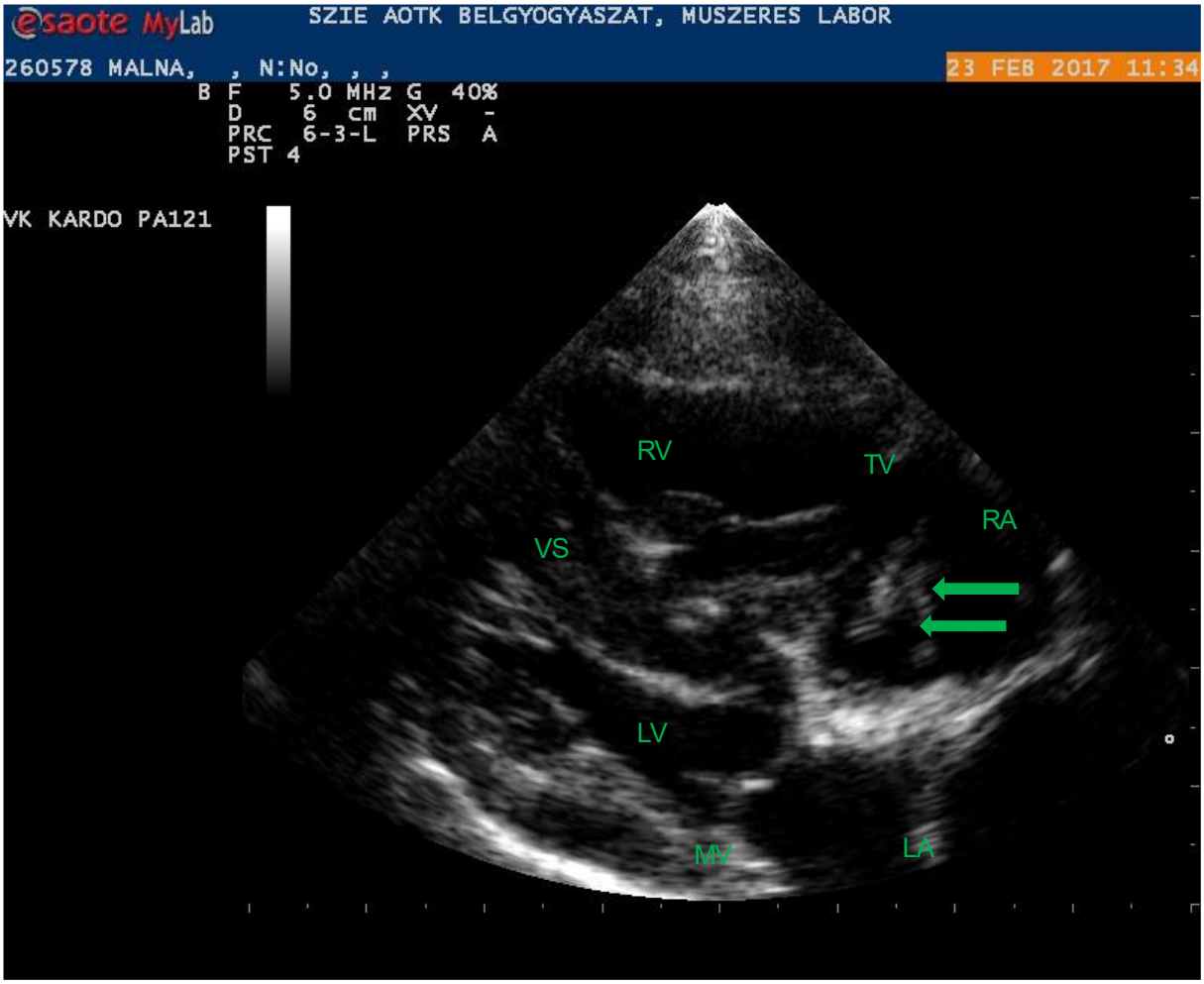


Figure 5. Cross-sections of adult heartworms (arrows) in the right atrium of a dog with caval syndrome (Clinical stage 4). Two-dimensional echocardiographic image. Right parasternal long axis plane four-chamber view. RV: right ventricle, RA: right atrium, TV: tricuspid valve, VS: ventricular septum, LV: left ventricle, MV: mitral valve, LA: left atrium. (Courtesy of Dr. Károly Vörös.)

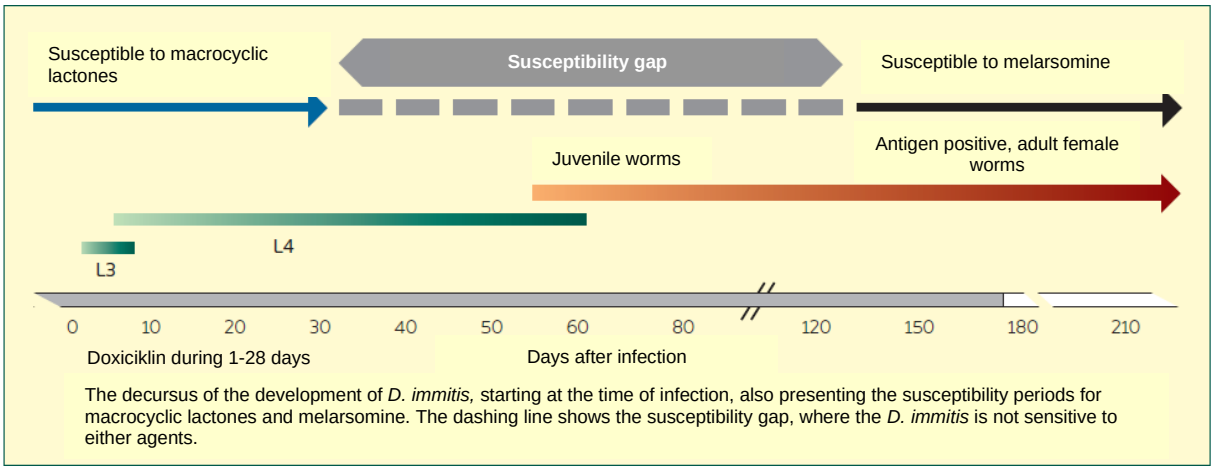
Visualization of adult heartworms in the main pulmonary artery or in the right heart can provide a definitive diagnosis. The body wall of the adult heartworms is highly echogenic and appears like “equal sings” (Venco et al. 2003, Túri and Hetyey 2014, Nelson et al. 2014, 2020). However, the exact number of heartworms could not be assessed, because adults locating deeper in the pulmonary arteries, within the lungs are not visible. Exact counting of the adult heartworms is not possible even in the main pulmonary artery. Namely, the same parasite could be visualized multiple times as its cross-sections, because of their location and body conformation. It is also important to mention, that the absence of adult HWs during echocardiography does not rule out the disease.

Electrocardiography (ECG) is applied less frequently as arrhythmias are rare in HWD, even in severe cases. Signs of right atrial and ventricular enlargement might be present on the ECG recordings in advanced HWD (Lombard and Ackerman 1984).

Treatment of HWD

The American Heartworm Society (AHS) recommends the complex, three-dose alternate melarsomine protocol for the treatment of HWD. This therapeutic schema includes a macrocyclic lactone (ML) four-times monthly against microfilariae as well as L3 and L4, a 28-day long application of doxycycline against *Wolbachia*, and a 3-dose regimen of melarsomine dihydrochloride for destroying adult heartworms (Nelson et al. 2014, 2020).

The different stages of the life cycle have different susceptibility to the macrocyclic lactones and melarsomine used during the complex therapy. This is the so-called susceptibility gap (**Figure 6**). During this period, mf and L4 larvae are destroyed, while L5 larvae develop further until they become susceptible to melarsomine (Nelson et al. 2020).



The decursus of the development of *D. immitis,* starting at the time of infection, also presenting the susceptibility periods for macrocyclic lactones and melarsomine. The dashing line shows the susceptibility gap, where the *D. immitis* is not sensitive to either agents.

Figure 6. The schematic of the complex therapy of heartworm disease. Based on Nelson et al. 2014, Farkas and Vörös 2015.

For heartworm prevention, MLs i.e., selamectin, ivermectin, moxidectin, and milbemycin oxime are approved by the Food and Drug Administration (FDA) (Nelson et al. 2014, 2020). As to Nelson et al. (2020) prevention of HWD should be started as early as possible, namely, no later than 8 weeks of age. A comprehensive list of prevention protocols can be found in the ESDA Guideline (2017) addressing puppies as to their age from 2 months to more than 7 months, depending on the suggestion of the manufacturers as well as history of the dogs.

Till the time of writing my thesis, only ivermectin has been used as part of the complex treatment protocol as recommended by the AHS. The combination of doxycycline and

ivermectin eliminates the L3 and L4 better, than doxycycline alone (Bazzocchi et al. 2008). The use of this combination before the three-dose melarsomine injection reduces the respiratory complications and heartworm related deaths (Nelson 2012, Nelson et al. 2017). There is a publication evaluating the outcome of the three-dose alternate therapeutic regimen including ivermectin (Maxwell et al. 2014). Topical moxidectin is approved by FDA to eliminate microfilariae for prevention of HWD. In a study on experimentally infected dogs, no adverse reaction was reported when microfilaremia was treated with moxidectin (McCall et al. 2014). Selamectin has a mild and lengthened effect against microfilariae compared to the other MLs used for HW prevention. Therefore, the chance of an adverse effect is considerably low even in microfilaremic dogs. However, selamectin is not applied as a part of the complex HWD treatment, probably because it would lengthen the duration of the therapy.

Melarsomine is highly effective against adult HWs, and it is the only adulticide drug approved by the FDA and recommended by the AHS given as intramuscular injection (Nelson et al. 2014, 2020). Due to the potential local (e.g., irritation, swelling or pain) and systemic posttreatment side effects, this drug should be applied cautiously, with strict adherence to the instructions of the manufacturers (Boehringer Ingelheim Immiticide® 2020, Zoetis, Inc. Diroban® 2020). Melarsomine should be injected deep intramuscularly in the epaxial muscles, at the region between the third and fifth lumbar vertebrae. Pulmonary arterial thromboembolism can occur during the spontaneous death of HWs as to the defensive mechanism of the dog. However, posttreatment thromboembolism (PTE) is even expected after successful adulticide therapy. This is elicited by the dead heartworms, which collapse, maybe fragmented, and are forced by the blood flow into the small, distal pulmonary arteries. The clinical sequelae of PTE can be minimized by strict exercise restriction during and after treatment (Rawlings et al. 1983, Bowman and Atkins 2009, Nelson et al. 2020).

Doxycycline is an antibiotic used against the *Wolbachia* bacteria, that contributes to kill *D. immitis* at all life stages (Kramer et al. 2008, Kramer and Genchi 2014, Nelson et al. 2017). However, gastrointestinal side effects can occur during the application of this drug (Schulz et al. 2011, Savadelis et al. 2018). In human and canine medicine, probiotics are found to be beneficial to prevent dysbacteriosis and restore the bacterial flora when using antibiotics (Grzeskowiak et al. 2015, Zimmermann and Curtis 2019, Schmitz 2021). As I made a search on the literature, I didn’t find any articles on the combination of doxycycline and probiotics regarding the therapy of HWD or other canine diseases.

Aims of the study

My dissertation covers three topics which goals are as follows:

1. In the first part of the thesis, we demonstrated the difficulties and determined the possibilities of serodiagnostic methods in infected dogs living in regions where concomitant infections by *D. immitis* and *D. repens* occur. The reason of this research was that we did not find information on the validation of *D. immitis* Ag tests in dogs from these regions. The VetScan Ag test was used as a model for Ag tests and the PCR assay served as a standard for the parasitological diagnosis. Sensitivity and specificity were studied of this Ag test under these conditions. As a second goal, we aimed to demonstrate if occult dirofilariosis could be the explanation for positive Ag tests despite of a negative PCR result of *D. immitis* what we experienced sometimes in our clinical praxis. In cases with occult dirofilariosis, several antigen tests of different manufacturers were applied for the diagnosis. These was based on the hypothesis that the chance for a cross-reaction among *D. immitis* and other helminths (including *D. repens*) is less when more than one HW Ag tests provide positive results.
2. As mentioned above, the standard therapy of HWD is recommended by the AHS which is effective and accepted internationally. This complex therapeutic protocol includes a macrocyclic lactone (ivermectin till nowadays to destroy mf as well as L3 and L4), doxycycline against the symbiotic *Wolbachia* bacteria, and the adulticide melarsomine dihydrochloride. However, side-effects and complications might occur of using these drugs. In our related research we have modified and complemented the AHS protocol with some additional medications and therapeutic measures to decrease the possible side-effects and to further improve the efficacy and outcome of the complex treatment. For these purposes, moxidectin was used instead of ivermectin as part of the complex treatment. Before our research, there has been only one preliminary report on the application of moxidectin with the contribution of one of my supervisors. These authors only partially used three-dose alternate melarsomine protocol (Bagi et al. 2017). In our study, probiotics were added to the application of doxycycline, in order to decrease the potential gastrointestinal side-effects of this antibacterial drug. The patients were sedated with butorphanol before the intramuscular application of melarsomine injection. The exact place of the needle was determined with the help of ultrasonography.
3. Occult HWD has not been published previously in Hungary. In a case report, we have described the diagnostic workup and the treatment of a severe case, for the Hungarian

readers. This part of my thesis also serves for demonstration of the clinical use of the scientific results of Chapter one and two.

1. Serodiagnostic difficulties and possibilities of heartworm disease in regions where both *Dirofilaria immitis* and *Dirofilaria repens* infections occur[1]

Introduction

Heartworm disease (HWD) is a worldwide distributed parasitosis caused by *Dirofilaria immitis* being present in Europe, America, Australia, and Asia. Several regions of the Central and Southern European countries are enzootic (McCall et al. 2008, Morchón et al. 2012a, Simon et al. 2012). Until 2000, HWD had been diagnosed in Hungary only in dogs imported from the USA (Boros et al. 1982, Vörös et al. 2000). The first autochthonous *D. immitis* infection was detected in a Hungarian Vizsla from the eastern part of Hungary (Jacsó et al. 2009). Since then, some papers have been published about dogs and wild canids infected with *D. immitis* in the country (Farkas et al. 2014, Tolnai et al. 2014, Bacsadi et al. 2016a, Trájer et al. 2016, Farkas et al. 2020). The other *Dirofilaria* species, *Dirofilaria repens* is present in Africa, Europe, and Asia, but not in the Americas (Simon et al. 2012, Genchi and Kramer 2017). The occurrence of this parasite has been known in Hungary for many decades (Fok et al. 1998, Széll et al. 1999, Jacsó and Fok 2006, Jacsó 2014, Farkas et al. 2020) as well as in other countries of Europe (Capelli et al. 2018).

D. immitis can cause fatal cardiorespiratory consequences and even death without proper treatment, whilst *D. repens* infection is less pathogenic. Their differentiation is especially important during the diagnostic work-up of clinical cases in regions where both helminths occur (ESCCAP Guideline 05 2019). For detecting microfilariae (mf) of both *Dirofilaria* species, the modified Knott test is used mostly (Jacsó and Fok 2006, Majoros and Juhász 2015a, Genchi et al. 2018). However, the Knott technique is rather applicable for diagnosing microfilaraemia without definitive distinction of *D. immitis* and *D. repens* (Magnis et al. 2013).

The polymerase chain reaction (PCR) technique as a molecular biological method can be considered as the most sensitive and reliable diagnostic method for *in vivo* diagnosis of blood filarioids (Favia et al. 1996, Rishniw et al. 2006, Latrofa et al. 2012, Rojas et al. 2015). This method allows definitive determination and distinction of *D. immitis* and *D. repens* if there are at least 4 mf in 1 ml peripheral blood (Gioia et al. 2010).

[1]This chapter is based on Becker, Zs., Holló, N., Farkas, R., Gyurkovszky, M., Reizigel, J., Olaszy, K., Vári, Z. and Vörös, K. (2022): Serodiagnostic difficulties and possibilities of heartworm disease in regions where both *Dirofilaria immitis* and *Dirofilaria repens* infections occur. Acta Vet. Hung. **70,** 92-99.

The circulating antigens are produced only by the adult female *D. immitis* worms (Roth et al. 1993, Goodwin 1998, Atkins 2003). The sensitivity and specificity of the serological methods are affected by some factors, e.g., by the number of adult female worms and by the amount of the circulating antigens (Roth et al. 1993, Lee et al. 2011, Aron et al. 2012, Burton et al. 2020). The application of the Ag tests has its distinguished importance in cases of occult dirofilariosis (more precisely occult heartworm infection) when no mf can be detected in the peripheral blood despite a persisting HW infection with the presence of adult heartworms. Occult dirofilariosis can have various causes, e.g., previous macrocyclic lactone application for prevention or unisex infections. Another important phenomenon is the so-called true occult dirofilariosis, which occurs when the mf are eliminated from the peripheral blood by the immune system of the infected dog (Wong et al. 1973, Rawlings et al. 1982, Bagi et al. 2017). For occult dirofilariosis, the PCR assay is not indicated as *D. immitis* DNA is extracted from mf in case of *in vivo* determination. Therefore, the diagnostic accuracy of the Ag tests is of utmost importance in occult dirofilariosis, as the sole laboratory diagnostic method in these cases (Nelson et al. 2010). This is especially true in those regions, including several European countries, where both *D. immitis* and *D. repens* may occur in infected dogs either separately or simultaneously (Ionica et al. 2015, Ciuca et al. 2016a, b, Bagi et al. 2017, Farkas et al. 2020).

We have found only a few publications reporting on the application of these Ag tests in those regions where both *D. immitis* and *D. repens* are present (Ionica et al. 2015, Ciuca et al. 2016a, b). Genchi et al. (2018) have reported their experiences regarding the sensitivity and specificity of the following Ag tests: Speed Diro™(BVT-Virbac); PetChek® HTWMPF (IDEXX); Witness Dirofilaria Ag Test (Zoetis); Idexx SNAP Heartworm RT test (IDEXX). However, Genchi et al. (2018) have not compared their Ag test results with molecular biological (i.e., PCR) methods to identify circulating mf but rather performed morphological analysis by using the Knott method. Trájer et al. (2016) and Farkas et al. (2020) have described the detection of occurrence of *D. immitis* and *D. repens* alone or concomitantly in Hungary. They used PCR without comparison with the serological diagnostic methods. It is also worth to mention that no serological tests are available to detect adult *D. repens* worms (Tarello 2011, Jacsó 2014, Genchi and Kramer 2017).

The VetScan Ag test (Abaxis VetScan® Canine Heartworm Rapid Test Kit) is an immunochromatographic sandwich assay test for the detection of adult *D. immitis* worms (Lee et al. 2011). Its performance in terms of sensitivity and specificity has been compared with those of numerous similar Ag tests (Atkins 2003, Lee et al. 2011, Aron et al. 2012, Rojas et al.

2015, Henry et al. 2018). However, these reports included canine populations from the Americas where *D. repens* does not occur.

In literature, we did not find reports on the validation of *D. immitis* Ag tests in dogs from regions where both *Dirofilaria* species are present. Therefore, the first goal of this study was to determine the sensitivity and specificity of the VetScan Ag test – as an example for Ag tests - in dogs originating from *D. repens* affected areas i.e., dogs with known single *D. immitis* or *D. repens* or with mixed infections. For these purposes, the PCR assay served as the most accurate method of our study. The second goal was to demonstrate if occult dirofilariosis could be the explanation for positive Ag tests despite of a negative PCR result of *D. immitis*. By answering these questions, we intended to demonstrate the diagnostic difficulties and possibilities of HWD in regions where concomitant infections by *D. immitis* and *D. repens* as well as occult dirofilariosis are present.

Materials and methods

Altogether 71 dogs were included retrospectively into the study. These dogs were randomly selected from dogs arriving for HW screening examinations or with clinical suspect of HWD. The examinations were done at the Clinic of Internal Medicine of the University of Veterinary Medicine Budapest (UVMB), Hungary, at the Budatétény Animal Hospital, Budapest, Hungary, and the Rákosliget Small Animal Ambulatory Clinic, Budapest, Hungary. Selection criteria included performed PCR assays, with positive results of *D. immitis* and/or *D. repens* as well as VetScan Ag test studies to detect adult female HWs.

EDTA preserved peripheral blood taken by the veterinarians of the clinics was examined freshly or preserved at 4°C for maximum two days. Blood samples were taken at different times of the day. These timings were not recorded during the study. The modified Knott test was applied at the Department of Parasitology and Zoology, UVMB to detect circulating mf (Majoros and Juhász 2015a, Majoros and Juhász 2015b).

DNA was isolated from 0.2 mL of blood from each sample using NucleoSpin® Tissue kit (Macherey-Nagel GmbH, Germany). Multiplex and conventional PCRs targeting fragments of 12S rDNA of both *Dirofilaria* spp. and 16S rRNA of *D. immitis* were used, respectively (Liu et al. 2005, Gioia et al. 2010). The majority of the PCRs were done at the Department of Parasitology and Zoology, UVMB, whilst some of them have been performed at the Praxislab Kft and at the Vet-Med-Labor Zrt. in Budapest, Hungary, where similar PCR assays were

applied. PCRs were done in 68 dogs with microfilaraemia detected by the modified Knott test. Three dogs were examined by PCR despite the negative Knott test, as they had a positive VetScan Ag test result, and their history and clinical findings suggested the possibility of HW infection. This was done keeping in mind that the PCR is more sensitive than the Knott test, i.e., it can be positive in Knott negative cases (Gioa et al. 2010, Rojas et al. 2015).

All samples were tested for the presence of *D. immitis* antigen with the VetScan Ag test (Abaxis VetScan® Canine Heartworm Rapid Test Kit, ABAXIS Europe GmbH, Germany). In addition to a positive VetScan Ag test, serologic test kits from other manufacturers were used in six dogs, which had negative PCR results for *D. immitis,* but positive for *D. repens,* in order to define the possibility of an occult dirofilariosis. To this end, the following Ag tests were applied on the same blood samples: DiroCHEK® Canine Heartworm Antigen Test Kit (Synbiotics Corporation, San Diego, USA); Witness Dirofilaria Ag Test (Zoetis Hungary); Idexx SNAP Heartworm RT test, (IDEXX Germany). The Ag tests were done from serum samples in the laboratory of the Department of Parasitology and Zoology, UVMB or from whole blood at the Department and Clinic of Internal Medicine, UVMB as well as at the two external clinics according to the prescriptions of the manufacturers.

Statistical methods. The sensitivity and the specificity of the VetScan Ag test were estimated in comparison to PCR results. Thus, only those cases were included in the analysis where VetScan Ag test as well as PCR results were available. The examined patients were selected as described above, therefore sensitivity and specificity of the VetScan Ag test is related to this population. The sensitivity was calculated by including cases with positive *D. immitis* results. The specificity was determined by using patients with negative *D. immitis* and positive *D. repens* PCR results as detailed in Results. Point estimates of sensitivity and specificity (Stevenson 2008) were accompanied by 95% Blaker confidence intervals (CI) (Blaker 2000).

Results

The dogs were divided into three groups based on the PCR results: Group 1: 26 (36.6%) dogs were positive only for *D. immitis*. Group 2: 21 (29.6%) dogs proved to be positive for both *D. immitis* and *D. repens*. Group 3: 24 dogs (33.8%) were positive only for *D. repens.*

The sensitivity of the VetScan Ag test was 46/47=97.7% (95% CI 89.0-99.8%) based on the compiled results of Group 1 and 2 where the dogs had PCR-proven *D. immitis* infections. For

the specificity of the VetScan Ag test, the results from Group 3 were used, in which dogs were *D. immitis* negative and *D. repens* positive by PCR. The specificity of the test was 19/24=66.7% (95% CI 45.6-83.1%).

Examining only Group 1 (n=26), - where all animals were positive only for *D. immitis* with PCR - the VetScan Ag test gave positive reactions in all cases except one dog. As such, the sensitivity in this group was 25/26 = 96.2% (95% CI 81.7-99.8%). When analyzing Group 2 (n=21), where all animals were PCR positive both for *D. immitis* and *D. repens*, the sensitivity within this group was 100% (95% CI 84.8-100%).

The sensitivity and the specificity of VetScan Ag test results were compared with previous studies performed in the USA where *D. repens* does not occur (**Table 2)**.

	Present study	**Atkins 2003**	**Aron et al. 2012****	**Henry et al. 2018**
Sensitivity % (95% CI)	97.7 (89.0-99.9) n=47	78.0 (72.0-84.0) n = 208	92.0 n=25	98.5 (95.7-99.7) n=200
Specificity % (95% CI)	66.7 (45.6-83.1)* n=24	97.0 (84.0-100) n = 32	100 n=24	94.0 (83.4-98.7) n=50

*Table 2 Comparison of the sensitivity and specificity values of the VetScan Ag test obtained in our study with those published by others. CI: Blaker 95% confidence interval; n: number of tested animals; *: Applied to patients found infected with D. repens and negative for D. immitis by PCR. **: CI was not provided.*

The sensitivity of the used Ag test, calculated based on the *D. immitis* PCR positive samples of Group 1 and Group 2, was excellent (97.7% in average). These results were similar to those of other authors as shown in **Table 2.**

The specificity of the Ag test, calculated based on Group 3 with only PCR positive *D. repens* samples, was lower compared to the reports from the USA. Atkins (2003) examined blood samples of dogs infected experimentally with known number of adult female HWs. Aron et al.

(2013) as well as Henry et al. (2018) verified the Ag positivity of their blood samples by necropsy. However, *Dirofilaria* PCR detections were not performed in these previous studies.

The samples of 6 dogs in Group 3 were positive with VetScan Ag test, despite that their PCR results were negative for *D. immitis* and positive only for *D. repens*. Of these 6 cases, 4 dogs were also seropositive when the Witness Dirofilaria Ag test was done additionally. The other 2 dogs were tested also with the Witness Dirofilaria Ag test, furthermore with the Idexx Snap Heartworm RT test, as well as with the DiroCHEK® Canine Heartworm Ag test, and all gave positive results.

Discussion

Several publications can be found in the literature on the evaluation of various Ag tests detecting HW infections. The sensitivity of these tests varies between 77% and 100%, while the specificity yields between 90% and 100% (Atkins 2003, Aron et al. 2012, Starkey et al. 2017, Genchi et al. 2018, Burton et al. 2020). As such, they are important parts of HW screening examinations and the diagnosis of HWD. However, it is known that Ag tests of *D. immitis* infections may give false positive reaction for *Spirocerca lupi* (Aroch et al. 2015), *Angiostrongylus vasorum* (Schnyder and Deplazes 2012), and *Acanthocheilonema dracunculoides* (Szatmári et al. 2020). False positive cross-reaction may also occur for *D. repens*. Genchi et al. (2018) did not find cross-reaction when they used several Ag tests in 21 cases, which were microfilaremic with Knott test. However, there was no PCR examination in that study. Ciuca et al. (2016b) performed PCRs to detect *D. immitis* and *D. repens* on 108 Knott positive canine blood samples as well as DiroCHEK®, Zoetis Ag test to demonstrate the presence of adult HWs. The antigen testing for *D. immitis* showed conflicting results in the examined 12 dogs with only *D. repens* mf. Two dog samples (16%) were antigen negative both before and after the preheat treatment of blood sera. Six dog samples (50%) demonstrated antigen positivity after heating, and four samples (30%) were antigen positive both before and after pretreatment. As to the hypothesis of Ciuca et al. (2016b), these results might suggest that dogs infected with *D. repens* can produce false *D. immitis* positivity, especially after preheat treatment, or may have also an occult infection with *D. immitis*.

D. repens cross-reaction with HW Ag tests has been indirectly proven by Venco et al. (2017). They reported on two experiments in the same publication: One study included live adults of *D. immitis, D. repens, Toxocara canis, Toxocara cati (*syn. *T. mystax), Dipylidium caninum,*

Taenia taeniaeformis, and *Mesocestoides* spp. larvae which were washed and incubated with saline solution. These solutions containing excretory/secretory antigens were tested with different HW Ag tests. In their second study, sera from dogs with natural infections by *An. vasorum* or *D. repens*, living in areas free of heartworm disease, were tested with the same Ag tests before and after preheat treatment. They found that cross-reactions with *An. vasorum* and *D. repens* might happen, and it can even potentially occur also with other helminths. *D. repens* appeared to release more antigens – responsible for cross-reaction – than the other worms examined. Preheat treatment reduced the specificity of the test by increasing cross-reactivity.

False negative Ag test results might also occur, mainly due to the low number of adult female heartworms, which can influence sensitivity (Atkins 2003). It is known that the negative Ag test may became positive, after heating the blood sample (Velasquez et al. 2014, Drake et al. 2015, Ames et al. 2017, Bendas et al. 2017, Savadelis et al. 2017). Other authors have also found that the occurrence of false positive reactions increased with sera preheating, thereby heating seems to decrease specificity (Venco et al. 2017, Savadelis et al. 2018, Gruntmeir et al. 2020). Sobotyk et al. (2021) performed *D. immitis* Ag test with DiroCHEK® Canine Heartworm Ag test on dogs experimentally infected with *D. repens*. No cross-reaction (i.e., false positivity) was found in any dogs prior to preheat treatment, whilst the antigen test became positive after heat treatment of the samples. They concluded that the preheat treatment of the serum possesses limited clinical value and should be applied with caution. The heating of blood samples is not recommended by the American Heartworm Society (AHS) for routine heartworm screening (Nelson et al. 2014, 2020, ESDA Guideline 2017, ESDA Guideline 2017). In addition, preheating is not included in the procedure manual of any manufacturers. For these reasons, we did not perform the preheat Ag test in our cases.

In the present study, we used the VetScan heartworm Ag test as an example for the in-clinic antigen tests. The results of this Ag test were compared with PCR results of known *D. immitis* and/or *D. repens* infected dogs. The sensitivity of the VetScan Ag test performed on *D. immitis* positive dog samples (Group 1 and 2) was excellent, in correlation to the relevant literature (Atkins 2003, Aron et al. 2012, Henry et al. 2018). However, its specificity was lower in Group 3, where dogs were infected with *D. repens* but negative for *D. immitis*, compared to the relevant data of these reports, all from the USA where *D. repens* does not occur.

In our study, *D. immitis* and *D. repens* infection occurred alone or as mixed infection in a similar proportion regarding the number of cases within the three groups. This observation was confirmed by PCR albeit it differs from the observations of others. Previous studies reported

lower rate of mixed infections than the rate of *D. immitis* or *D. repens* cases alone (Ionica et al. 2015, Ciuca et al. 2016a, b, Trájer et al. 2016, Farkas et al. 2020). Ionica et al. (2015) screened 390 dogs with a multiplex PCR assay. Of them, 46 (11.8%) were positive for dirofilariosis and 8 (2%) for *Acanthocheilonema reconditum* infection. In their study, coinfections with *D. immitis* and *D. repens* were found in 11 (23.9%) positive dogs and those with *D. repens* and *Ac. reconditum* in 2 dogs (4.3%). Ciuca et al. (2016a) examined 45 stray dogs with Knott test and 23 (51.1%) patients were positive for circulating mf. Nineteen dogs were positive for *D. immitis* and 4 for both *D. immitis* and *D. repens* using another type of multiplex PCR system. Ciuca et al. (2016b) have performed PCR examination using the same multiplex assay on 24 dogs, which were positive for circulating mf. Six out of 24 dogs have been found infected with *D. immitis*, 12 with *D. repens* and 6 animals with both *D. immitis* and *D. repens*. In one of these cases with „mixed" dirofilariosis, *Ac. reconditum* infection has also been detected. Based on the positive DiroCHEK® Canine Heartworm Ag test before and after heat treatment of the samples, the authors have hypothesized that dogs being infected with *D. repens* might had also an occult infection with *D. immitis*. Similar hypothesis has been suggested by Rojas et al. (2015) who found *D. immitis* positivity with the Vetscan Ag test in five dogs negative by both the Knott test and PCR.

One of the explanations of the lower specificity obtained in our study could be the possible occurrence of occult dirofilariosis (i.e., occult heartworm disease). We suspected occult infections for 6 dogs in Group 3 which were *D. immitis* negative with PCR, but positive for *D. immitis* antigen with several Ag tests of different manufacturers. The *in vivo* proof of occult dirofilariosis could be the demonstration of adult heartworms in the main pulmonary arteries during echocardiography. However, no adult heartworms were detected by this imaging method in these dogs. The explanation could be that these cases of our study belonged to the 1st and 2nd clinical categories of HWD, where adult worms rarely occur in those vessels (Bowman and Atkins 2009, Nelson et al. 2020). Nevertheless, our results seem to provide a stronger argument for the coinciding presence of *D. repens* infection and occult dirofilariosis compared to those of Ciuca et al. (2016b) and Rojas et al. (2015), as these authors applied only one Ag test in their studies. Ionica et al. (2015) also performed only one Ag test and found 7 Ag-positive cases with sole *D. repens* infection i.e., without *D. immitis* PCR positivity. In our opinion, the chance for a cross-reaction caused by *D. repens* is less when several Ag tests are applied, and all are positive. This phenomenon rather speaks for occult dirofilariosis as it is supposed in our cases. The same scenario might be valid to exclude potentially false positive cross-reactions with other

filarioid worms like *Spirocerca lupi* (Aroch et al. 2015) and *An. vasorum* (Schnyder and Deplazes 2012).

In those continents/countries where both helminths occur, the parasitological diagnosis of *D. immitis* including its differentiation from a sole *D. repens* infection is inevitable before starting any therapy. In dogs with a positive Knott test demonstrating microfilaraemia, PCR should be performed to identify these parasites with great certainty (Favia et al. 1996, Rishniw et al. 2006, Latrofa et al. 2012, Rojas et al. 2015). However, Ag tests are also important, especially in occult dirofilariosis cases (Vörös et al. 2017, Genchi et al. 2018, Nelson et al. 2020). This issue raises significant diagnostic dilemmas when a decision should be made for the treatment of HWD (Maxwell et al. 2014, Nelson et al. 2020). At the Clinic of Internal Medicine of UVMB, we use three or sometimes even four Ag tests from different manufacturers simultaneously in cases of occult dirofilariosis (Vörös et al. 2017). The complex treatment is applied only if all tests are positive and/or adult HWs are seen in the main pulmonary arteries. However, in the *D. immitis* PCR-negative cases, even multiple positive Ag tests cannot fully exclude the possibility of a false positive cross-reaction with other blood filaroids. Nevertheless, it can be assumed that the proper treatment of HWD is better than not to perform it because of the lack of an all-round diagnostics (Nelson et al. 2020). In our patients with equivocal results, we offer the moxidectin-doxycycline therapy i.e., without melarsomine as it has been recently described (Ames et al. 2017, Bendas et al. 2017, Savadelis et al. 2017, Genchi et al. 2019). Bendas et al. (2017) found, that dogs treated with moxidectin, and doxycycline could become free from adult heartworms earliest 6 months after the beginning of the therapy, and all of their patients were antigen free after 18-24 months. Savadelis et al. (2017) detected no mf after 21 days posttreatment and found the efficacy of the moxidectin/doxycycline combination in the elimination of mature *D. immitis* 95.9%. Ames et al. (2017) found that the combination protocol of moxidectin and doxycycline is well tolerated in most of the dogs with HWD (96%), They offered melarsomine therapy for the owners of 5 patients after 210 days of moxidectin - doxycycline treatment for HWD dogs still being antigen positive. Nevertheless, owners decided to continue the moxidectin and doxycycline treatment even in those dogs. However, from their 22 patients, 1 dog still had positive antigen test after 701 days. The AHS does not suggest protocols containing long-term macrocyclic lactone administration without melarsomine, due to the long treatment time, whilst HWD can progress, the necessity of long-term exercise restriction, and the potential of development of macrocyclic lactone resistance in HW subpopulations (Nelson et al. 2020).

The present study has several limitations. Unfortunately, there were no Ag-test-positive dogs with *D. repens* positive and *D. immitis* negative PCR results within the examined population, with echocardiographically detectable adult heartworms which could have been an *in vivo* proof of occult dirofilariosis. The PCR of blood samples, collected from the patients, were performed in three different laboratories for both financial and organizational reasons. Another limitation of the study is that we did not apply any methods to exclude *S. lupi* or *An. vasorum* infections. A cross-reaction with *D. repens* might be directly proven with *D. immitis* Ag tests on *D. repens* PCR positive dogs, then performing necropsy to exclude a simultaneous *D. immitis* infection. No such reports exist in the literature as far as I know. In our study, the occult dirofilariosis could not be confirmed definitely by post-mortem pathological examination, as all of these patients healed after successful treatment.

Conclusions

The sensitivity of the Ag test performed on *D. immitis* positive dog samples was excellent, compared to the previous reports on this subject. However, its specificity was lower in the examined dogs, which were infected with *D. repens* but negative for *D. immitis*, when compared to the related reports, all from the USA, where *D. repens* does not occur. In cases of positive *D. repens* and negative *D. immitis* PCR results, occult dirofilariosis could be the explanation for the positive *D. immitis* Ag tests. These observations highlight the importance of performing multiple Ag tests in those areas where both *Dirofilaria* species are present. This has special importance when a PCR cannot be performed in the lack of mf in the peripheral blood or when this technique reveals only the presence of *D. repens*. Further research would be required on a larger population to explore the occurrence rate of occult dirofilariosis, as well as the antigen cross-reaction caused by *D. repens* in cases of canine heartworm infections in these regions.

2. Application of moxidectin and ultrasound-aided injection of melarsomine during the American Heartworm Society recommended treatment protocol in *Dirofilaria immitis* infected dogs[2]

Introduction

Heartworm disease (HWD), which is one of the most important canine vector-borne diseases in Europe, America, Australia, and Asia, is caused by the nematode *Dirofilaria immitis* (McCall et al. 2008, Morchón et al. 2012a, Simón et al. 2012, Farkas et al. 2020, Fuehrer et al. 2021). It is an emerging parasitosis of dogs in many European countries including Hungary, mainly due to climatic changes being favorable for transmitting mosquitoes as discussed in the Introduction chapter of my thesis.

The other filarioid helminth of this genus, *D. repens* occurs in Africa, Europe (also in Hungary), and Asia, but not in the Americas (Fok et al. 1998, Széll et al. 1999, Capelli et al. 2018). *D. repens* infection is more common in people than *D. immitis* resulting in mainly ocular and subcutaneous diseases (Engelsberg and Bläckberg 2022, Wylęgałaet al. 2022).

Emerging importance of *D. immitis* in humans has also been reported (Laidoudi et al. 2021, Mendoza-Roldan et al. 2021). In people, the life cycle of *D. immitis* is not complete and the parasite usually elicits benign pulmonary nodules. These alterations are subclinical or cause only minor clinical symptoms. However, they have differential diagnostic dilemmas namely during thoracic radiography and computed tomography (Morchón et al. 2012a, Simón et al. 2012, Malik et al. 2016). Human subcutaneous and ocular dirofilariosis caused by *D. immitis* have also been described (Avellis et al. 2011, Falidas et al. 2016, Parsa et al. 2020). Proper diagnosis, treatment, and prevention of HWD is inevitable for protecting canine patients and to inhibit human infections which are transmitted by infected mosquitoes (Mendoza-Roldan et al. 2021).

The life cycle of *D. immitis* consists of different phases, which should be considered during its therapy (Hoch and Strickland 2008a, Bowman and Atkins 2009). All forms of the parasite live together with the endosymbiont *Wolbachia* bacteria (Kramer and Genchi 2014, Nelson et al.

[2]This chapter is based on Vörös, K., Becker, Zs., Kónya, R., Arany-Tóth, A. and Farkas R. (2022b): Application of moxidectin and ultrasound-aided injection of melarsomine during the American Heartworm Society recommended treatment protocol in Dirofilaria immitis infected dogs. Vector Borne Zoonotic Dis. **22,** 382-390. and Vörös, K., Becker, Zs., Dudás-Györki, Z., Gronover, B.S. and Szalay F. (2022a): Ultrasonography of the paralumbar muscles as a new aid during melarsomine treatment in canine heartworm disease. Acta Vet. Hung. doi:10.1556/004.2022.00034. Online ahead of print

2017). The details of the life cycle of the parasite are outlined in the Introduction chapter of my thesis.

The American Heartworm Society (AHS) recommends the three-dose alternate melarsomine protocol to treat HWD. This therapeutic regimen includes monthly application of a macrocyclic lactone (ML) through 4 months against microfilariae as well as L3 and L4, a 28-day course of doxycycline against *Wolbachia*, and a 3-dose regimen of melarsomine dihydrochloride for destroying adult heartworms (Nelson et al. 2014, 2020).

Among the macrocyclic lactones, selamectin, ivermectin, moxidectin, and milbemycin oxime are approved by the Food and Drug Administration (FDA) for heartworm prevention (Nelson et al. 2014, 2020). The AHS treatment protocol recommends the preventive dose to reduce new infections and eliminate susceptible larvae. Adverse (anaphylactic i.e., shock like) reactions are rare with these macrocyclic lactones at preventive doses, but caution is advised. Pretreatment with antihistamines and glucocorticoids minimize potential hypersensitivity reactions (Bowman and Atkins 2009, Nelson et al. 2014, 2020).

At present, ivermectin is the only ML that has been used in published studies as part of the AHS protocol. It has been shown that doxycycline/ivermectin eliminates *Wolbachia* better than doxycycline alone (Bazzocchi et al. 2008). Kramer et al. (2011) reported that dogs treated with ivermectin, doxycycline and melarsomine had a virtual absence of pulmonary thrombi. These were terminal studies that provided histopathology documenting these positive effects. To date, no one has conducted similar studies with moxidectin in place of ivermectin as to the available literature. Dogs receiving doxycycline and ivermectin prior to the three-dose melarsomine injections had fewer respiratory complications and heartworm-related death than dogs without doxycycline (Nelson 2012, Nelson et al. 2017). There is a publication evaluating the outcome of the three-dose alternate therapeutic regimen including ivermectin (Maxwell et al. 2014). Topical moxidectin is approved by (FDA) to eliminate microfilariae for prevention of HWD. In a study on experimentally infected dogs, no adverse reaction was reported when microfilaremia was treated with moxidectin (McCall et al. 2014). There is only a preliminary report on the application of moxidectin instead of ivermectin. These authors partially used three-dose alternate melarsomine protocol (Bagi et al. 2017).

Doxycycline is applied perorally to eliminate *Wolbachia* bacteria which contributes to the death of *D. immitis* at all life stages (Kramer et al. 2008, Kramer and Genchi 2014, Nelson et al. 2017). However, side effects might occur, the most common ones being gastrointestinal

disorders (anorexia, vomiting, diarrhea, and weight loss) (Schulz et al. 2011, Savadelis et al. 2018). During several oral antibiotic treatments, probiotics are added to prevent dysbacteriosis by restoring the bacterial flora and to help with antibacterial treatment in human and canine medicine as well (Grzeskowiak et al. 2015, Zimmermann and Curtis 2019, Schmitz 2021). When searching through the literature, I did not find publications on the combination of doxycycline and probiotics regarding the therapy of HWD or other canine diseases.

Melarsomine is highly effective against adult HWs, and it is the only adulticide drug approved by the FDA and recommended by the AHS given as intramuscular injection (Nelson et al. 2014, 2020). This drug, however, might have systemic and local posttreatment side effects and it should be applied with strict adherence to the directions of the manufacturers (Boehringer Ingelheim Immiticide® 2020, Zoetis Inc., Diroban®, 2020). Posttreatment thromboembolism (PTE) is expected after successful adulticide therapy. This is elicited by the dead heartworms, which collapse, maybe fragmented, and are forced by the blood flow into the small, distal pulmonary arteries. The clinical sequelae of PTE can be minimized by strict exercise restriction during and after treatment (Rawlings et al. 1983, Bowman and Atkins 2009, Nelson et al. 2020). According to the manufacturer's prescriptions, melarsomine should be injected only by deep intramuscular injection in the epaxial (lumbar) muscles in the 3rd through 5th lumbar region. Local side effects like irritation, swelling and pain at the injection site are common but usually mild. Persistent nodule formation can also occur, mostly without clinical consequences (Atkins and Miller 2003, Maxwell et al. 2014, Boehringer Ingelheim Immiticide® 2020, Zoetis Inc., Diroban® 2020). Rarely, severe swelling, sterile abscess or even progressive myelopathy can also develop. The later complication is possibly caused by improper restraint and application followed by ongoing sterile inflammation along the fascial planes or nerve roots into the epidural space (Hettlich et al. 2003, Moore et al. 2013). To minimize or even avoid local complications, it would be useful to further determine the exact location and depth of the injection needle, which is optimally the middle of the paralumbar musculature. Our research group published a report on an ultrasound method for imaging the intramuscular application of melarsomine (Vörös et al. 2022a). This technique proved to be useful before and after melarsomine injections to determine the injection site and to follow potential posttreatment local side effects.

The main goal of the present research was to complement the 2014 AHS therapeutic protocol with some additional therapeutical measures and supplementary medications, in order to improve the efficacy of the treatment with special regard by applying moxidectin instead of

ivermectin and to minimize the potential post-therapeutic complications of doxycycline and melarsomine.

Materials and methods

This prospective study involved 44 dogs treated at the Small Animal Clinic of the University of Veterinary Medicine, Budapest (SAC of UVMB) between July 2014 and March 2020. Written and signed owner's consent was obtained in each case. The implemented procedures were compliant with the guidance of the Animal Welfare Committee of the UVMB.

The modified Knott-test was applied to detect circulating microfilariae (Genchi et al. 2007). Circulating antigens of *D. immitis* were determined by the VetScan antigen test (Abaxis VetScan® Canine Heartworm Rapid Test Kit, ABAXIS Europe GmbH, Griesheim, Germany) as described earlier (Becker et al. 2022a). Multiplex and conventional PCR techniques were used in cases of microfilaremia to identify *D. immitis* and/or *D. repens* infections as published (Gioia et al. 2010, Farkas et al. 2020). In dogs without microfilaremia i.e., in occult dirofilariosis (Wong et al. 1973, Rawlings et al. 1982), PCR was not done. Therefore, the diagnosis of HW infection of these cases was based on the simultaneous positivity of at least two and maximum four antigen tests of different manufacturers as reported previously (Vörös et al. 2017, Becker et al. 2022a) and discussed in Chapter 1 and 3 of my thesis.

Data were collected about the history, signalment, and a detailed physical examination was done, especially on the cardiorespiratory system. Thoracic radiography and echocardiography were also applied. After that, the dogs were classified into the four pretreatment clinical stages as described in the Introduction chapter of this thesis. M-mode, two-dimensional, and Doppler echocardiography also served for determination of cardiological consequences of HWD and to detect adult HWs within the main pulmonary artery and in the right heart (Lombard and Ackerman 1984, Venco et al. 2003, Brown and Gaillot 2008).

Treatment

The modified and expanded therapeutic regimen applied in this study is summarized in **Table 3**, as compared to the original three-dose alternate melarsomine management protocol (Nelson et al. 2014). Dosages of the applied drugs are also indicated in **Table 3**. Patients of Clinical Stage 3 were stabilized first by symptomatic treatment, to treat right heart failure (Hoch and

Strickland 2008b, Ware 2014). In these cases, the therapy of HWD was started 10-14 days later, depending on the clinical status of the patient.

Day	**AHS recommended management protocol***	**Modifications and additional therapy of the present study****
Day 0	Begin exercise restriction. If the dog is symptomatic: stabilize with appropriate therapy and nursing care. Prednisone prescribed orally from day 1, at 0.5 mg/kg BID 1st week, 0.5 mg/kg SID 2nd week, 0.5 mg/kg EOD 3rd and 4th weeks.	*To prevent anaphylaxis* if mf detected: prednisolone prescribed orally from one week before day 1, applying the same therapeutic regimen for 4 weeks. *To prevent thromboembolism* possibly caused by mf: clopidogrel 2-4mg/kg/SID orally. To prevent GI side effects of prednisolone: famotidine 0.5-2.0 mg/kg SID (BID) po. or omeprazole 0.5-1.0 mg/kg SID (BID) po.
Day 1	Administer heartworm preventive*** If mf detected, pretreat with antihistamine and glucocorticoid if not already on prednisone. Observe for at least 8 hours for signs of anaphylaxis.	Heartworm preventive: moxidectin topically, applied as per package insert. *To prevent anaphylaxis* if mf detected: dexamethasone 0.1-0.2 mg/kg im. just before moxidectin application; chloropyramine 0.5-1.0 mg/kg im. 4 hours after moxidectin application.
Days 1-28	Administer doxycycline 10 mg/kg BID for 4 weeks.	*To prevent GI side effects of prednisolone and doxycycline* during day 1 to day 28: famotidine or omeprazole as written above. *To prevent GI side effects of doxycycline*: probiotics made for veterinary use during day 1 to day 28 as per package insert.
Day 30	Administer heartworm preventive.	Heartworm preventive: moxidectin topically.
Day 60	Administer heartworm preventive. First melarsomine injection 2.5 mg/kg im. Prednisone for 4 weeks as above.	Days 60, 90, and 91: Heartworm preventive: moxidectin topically. *Before melarsomine injection:* *Sedation* with butorphanol 0.2-0.4 mg/kg im.; *Ultrasound examination* of the epaxial (lumbar) muscles to determine the exact location of melarsomine injection. *Keeping the injection needle in place* for 5 minutes during injection to prevent leakage of melarsomine. *To prevent thromboembolism:* dexamethasone 0.1 (0.2) mg/kg im. before melarsomine inj.
Day 90	Administer heartworm preventive. Second melarsomine injection 2.5 mg/kg im.	
Day 91	Third melarsomine injection 2.5 mg/kg im. Prednisone for 4 weeks as above.	

		During the 60th to 69thdays: dalteparin sodium 100-150 IU/kg/SID sc. for 10 days.
Day 120	Test for presence of mf. If positive treat with a microfilaricide and retest in 4 weeks.	No modifications or additional measures.
Day 271	Antigen test 6 months after completion; screen for microfilariae.	No modifications or additional measures.

*Table 3. Comparison of the three-dose alternate melarsomine AHS management protocol with the therapeutic scheme of the present study. *: Nelson et al. 2014; **Dosages of prednisolone, doxycycline, and melarsomine were the same as indicated in the AHS protocol; ***: Only ivermectin as part of the AHS treatment was published earlier; mf: microfilariae; im.: intramuscularly; GI: gastrointestinal; po.: per oral; sc.: subcutaneously.*

Microfilaremic dogs were observed for 8 to 12 hours at the SAC of UVMB intensive care unit during the day of the 1st moxidectin application, with continuous supervision. No pretreatment therapy and hospitalization were provided for dogs without microfilaremia. Exercise restriction was advised during and after the treatment period as recommended (Nelson et al. 2014).

Hospitalization with cage rest was done for 24 and 48 hours on the 60th and 90-91st days during melarsomine treatment. After 12 hours starvation and 4-6 hours water deprivation, butorphanol was given intramuscularly for analgesia and sedation, 25 to 30 minutes before the melarsomine injection. The patients were kept in a quiet, dark room to provide calm surroundings during this period. Then, they were placed on the examination table in sternal recumbency and were gently but properly immobilized by two persons. The melarsomine injection was given in the epaxial (lumbar) musculature (in the 3rd through 5th lumbar region) in accordance with the manufacturer's prescriptions using the alternate left and right lumbar regions during the 60th, 90th, and 91st days (Merial Limited 2010).

The thickness of the paralumbar musculature and the exact place of the needle injection was determined by ultrasonography as described by Vörös et al. (2022a) and shown on **Figure 7**.

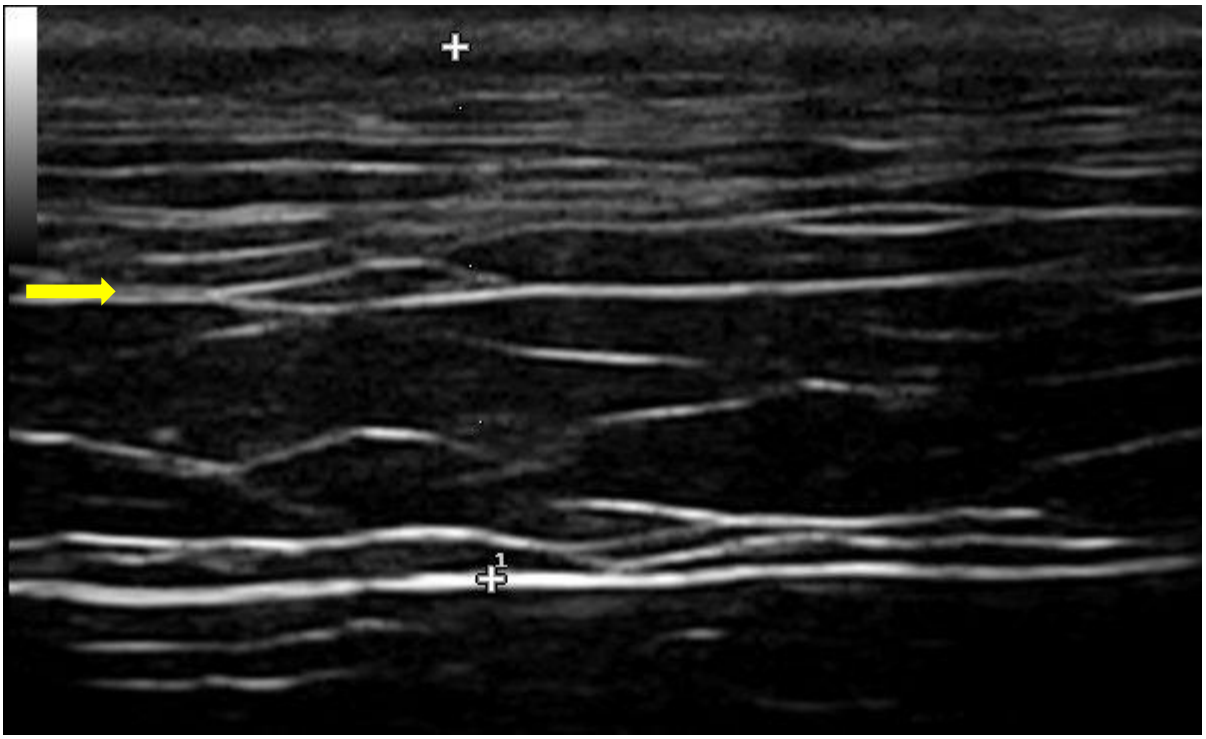


Figure 7. Physiologic, oblique-horizontal sonographic image, more or less perpendicular to the paralumbar muscles in dog with HWD before melarsomine injection. The echogenic lines represent the intramuscular connective tissue spaces of the musculature (thoracolumbar fascia). One of them is marked with an arrow. The + signs represent the measurement points.

After the injection of melarsomine, the needle was kept in place for 5 minutes to ensure absorption of the drug and to prevent its leaking into the tissues whilst pulling out the needle. Meanwhile, the patient was still immobilized by the assisting staff. Immediately thereafter, the site of injection was controlled by ultrasound examination (Vörös et al. 2022a). Water and food were supplied for the dogs only 2 and 4 hours after the injection procedure, respectively, because of the sedative effect of butorphanol, to avoid dysphagia. During each hospitalization event, the clinical status of the patients was monitored by physical examination.

Posttreatment systemic complications of melarsomine were checked by physical examination on the 61st, 90th, 91st, and on the 92nd days. In addition, owners and referring veterinarians were asked to provide information on the complications and the activities of the dogs by phone calls. Thoracic radiography was also performed in cases with respiratory symptoms. Local complications at the injection site were reported by the owners when they recognized painful behavior and/or swellings. In addition, physical examination of the affected epaxial lumbar musculature and ultrasonography were performed at each injection sites on the 61st, 90th, 91st, and on the 92nd days. The outcome of the treatment was checked by oral interviews either on

the 271st day when owners arrived at our clinic for the control Ag test, or by phone calls and/or by email correspondence when this Ag test was performed elsewhere. This timing was chosen as to the AHS recommendation issued at the time of starting this research (Nelson et al. 2014). However, Maxwell et al. (2014) found 6/18 patients being still antigen positive at this time. The explanation of this phenomenon is that dead HWs can still release antigen into the blood stream following worm death (Maxwell et al. 2014). In the latest AHS recommendation, control antigen testing is suggested even at the 365th posttreatment day (Nelson et al. 2020).

Results

Data of the dogs and their parasitological results

There were 24 female and 20 male patients in the population. Their age was 5.2 ± 3.1 years (range 1-13 years), and their mean body weight was 22.8 ± 13.6 kg (range 5-76 kg). Of the 44 patients, 19 (43%), 18 (41%) and 7 (16%) dogs were categorized in Stage 1 (mild), Stage 2 (moderate) and Stage 3 (severe), respectively. There were no dogs with caval syndrome, belonging into Stage 4.

Twenty-three out of 43 (57.5%) dogs had microfilaremia. The VetScan Ag test became positive in 43 (97.7%) cases. No microfilariae were detected in 21 dogs. PCR examinations were performed in 28 dogs, and 22 (78%) were positive for *D. immitis*, of which 8 patients were infected with both *D. immitis* and *D. repens*.

Therapeutic results

Pretreatment of Stage 3 cases. Additional symptomatic therapy was applied in 7 cases belonging to Stage 3 with favorable results, making possible to begin the complex heartworm treatment within 10-14 days. In a patient with right heart failure from Stage 3, the HWs disappeared from the right atrium and from the tricuspid valve region 10 days after cardiac treatment with furosemide, pimobendane, and potassium supplementation **(Figure 8 and 9**). In 2/7 of these dogs, the adult heartworms were not seen anymore within the main pulmonary artery, 6 and 10 days after the 1st application of moxidectin, respectively.

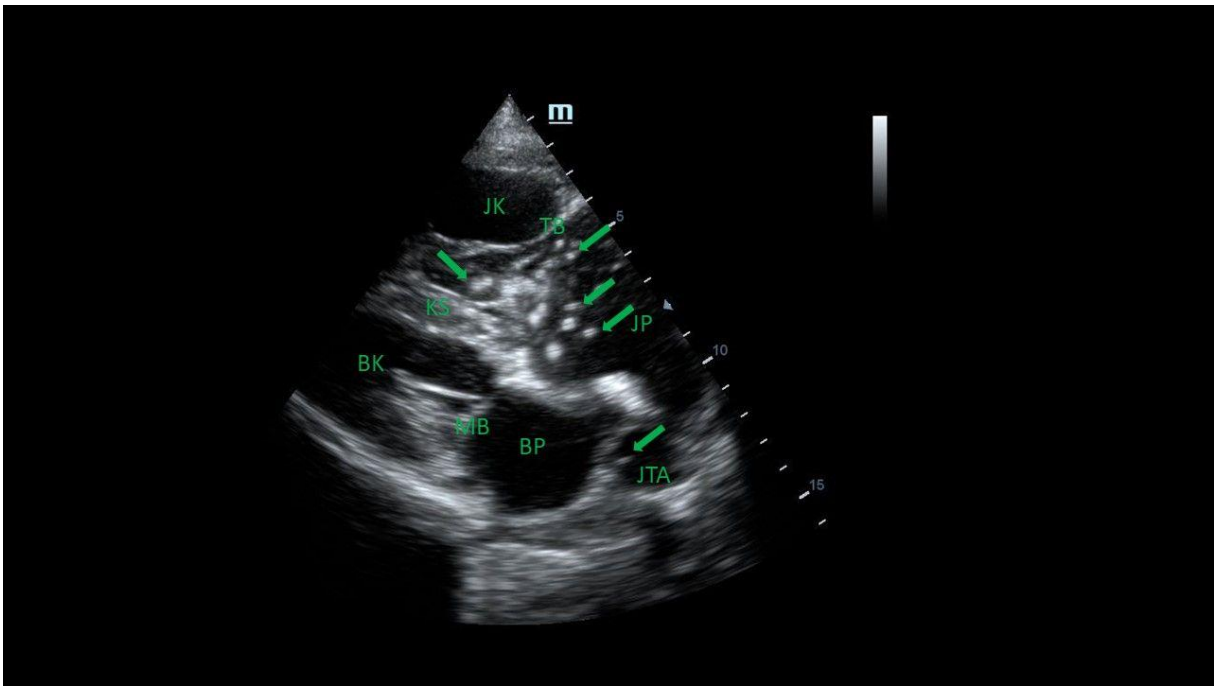


Figure 8. Severe (Stage 3) heartworm disease in a dog with right-side heart failure and with a grade 2/6 systolic, tricuspid cardiac murmur. On the pretreatment two-dimensional echocardiographic image, both the right atrium (JP) and the right ventricle (JK) are dilatated. There are several cross-sections of adult heartworms within the right atrium, at the tricuspid valve (TB), and even within the right ventricle, close to the tricuspid valve (arrows). One adult heartworm can also be seen within the dilated right main pulmonary artery (JPA) marked with an arrow. Right parasternal, long-axis image, four-chamber view. KS: interventricular septum; BP: left atrium; BK: left ventricle; MB: mitral valve. From Becker et al. 2022b. (With permission.)

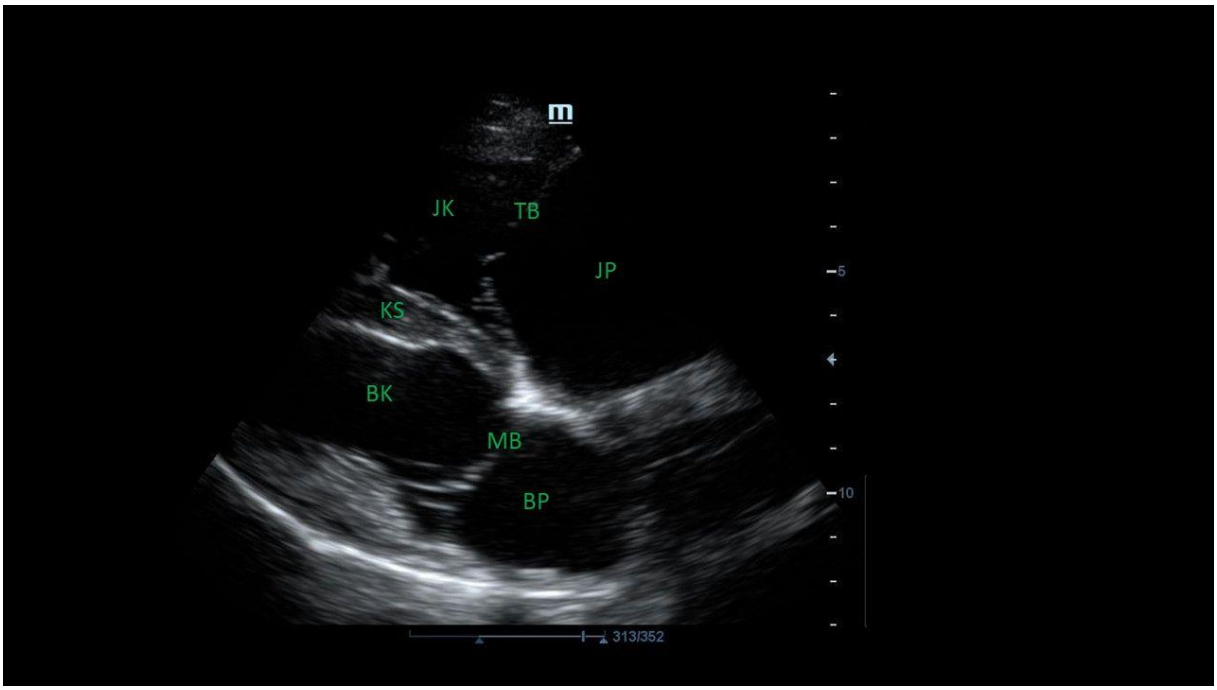


Figure 9. Two-dimensional echocardiographic image of the dog with severe (Stage 3) heartworm disease, shown on Figure 8, at the 10th day of the treatment of right-heart failure. The right atrium and the right ventricle are still dilated but no heartworms can be seen. Right parasternal, long-axis image, four-chamber view. TB: tricuspid valve; KS: interventricular septum; BK: left ventricle; BP: left atrium; MB: mitral valve. From Becker et al. 2022b. (With permission.)

Treatment of heartworm disease

No anaphylactic reactions occurred during any moxidectin applications, including the 23 dogs with microfilaremia. Adverse (side) effects of doxycycline were observed in 3 (6.8%) dogs. All these patients were anorectic, one dog vomited, and 2 patients had diarrhea. These symptoms healed after symptomatic therapy and by decreasing the dosage of the drug from 10 mg/kg BID to 5 mg/kg BID. The doxycycline therapy was not ceased in either case.

The butorphanol premedication of the patients provided proper sedation in 42 (95.4%) cases, while the dogs remained conscious during the whole procedure. They remained in place with minimal restriction but could walk after the melarsomine injection procedure. The mild sedation lasted for 1.5-2 hours. The other 2 dogs (4.6%) needed general anesthesia, with intravenous propofol, because of excitement and aggressivity, respectively.

The ultrasonographic examination and cross-sectional measurements of the paralumbar musculature allowed to plan the exact depth and site of the melarsomine injection. It was injected into middle of the epaxial muscles, that we confirmed by ultrasound after the

application. By keeping the injection needle in place for 5 minutes resulted that there was no detectable leakage of the melarsomine solution to the surface of the skin after the removal of the needle. We observed the distribution and absorption of melarsomine macroscopically, and it was dissolved from the injection site in 3 minutes as described (Gronover 2019, Vörös et al. 2022a).

Among the systemic posttreatment complications caused by melarsomine, vomitus was observed in one (2.3%) patient, temporary diarrhea occurred in 2 (4.6%) dogs, while pancreatitis was diagnosed in one (2.3%) case during the 1st and 2nd posttreatment days. These symptoms were cured by symptomatic treatment within a few days. Transient anorexia occurred in 2 (4.6%) cases, and transient polydipsia were reported by the owner in 1 (2.3%) dog. Both symptoms ceased spontaneously within some days. Coughing and mild dyspnea was observed in one dog (2,3%) which healed for symptomatic therapy in a few days. The comparison of our findings and two other studies are demonstrated in **Table 4**.

Side effect	Present study	Bagi et al. 2017	Maxwell et al. 2014
Diarrhea	4,6%	21%	ND
Vomitus	2,3%	12%	ND
Anorexia	4,6%	15%	ND
Pancreatitis	2,3	ND	ND
GI side effects*	9,2*%	ND	24%
Coughing	2,3%	21%	48%
Polydipsia	2,3	ND	ND
Dyspnea	2,3%	9%	14%
Death	0%	13%	14%

*Table 4. Comparison of the side effects found in the present study with two previous studies. Percentages are related to the whole numbers of patients. US: ultrasonography, GI: gastrointestinal. *: Sum of lines to 5. 2 ND: no data.*

Local reactions caused by the melarsomine injection were not found by physical examination in 13/44 (30%) dogs. In some patients, the injection site was found within the musculature only by the posttreatment ultrasound examinations. There were mild side effects in 29/44 (66%) cases. Within this latter group, the owners reported only mild pain for a few days in 8 dogs. A plum-sized, not painful swelling was found in 21 patients. These swellings did not interfere with the daily routine lifestyle of the patients, and they disappeared within 4-6 weeks in 2 dogs, without any therapeutic interventions. Just a small, firm nodule remained in 19 dogs. A severe, painful, apple sized swelling occurred in 2/44 (4%) cases after the first melarsomine

application. In these patients, it was not possible to use alternate lumbar sites for the consecutive two injections. Rather, two melarsomine injections were applicated on the same side from 2-3 cm apart during the 90th and 91st days.

Outcome

All 44 dogs healed clinically based on the physical examinations on the 120th day and on the reports of the owners. No microfilariae were detected in 33/44 patients with the Knott test on the 120th day. An Ag test was done in 33/44 dogs on the 271st day and all become negative.

Discussion

The main goals of our study were to apply the three-dose alternate melarsomine therapeutic protocol (Nelson et al. 2014) with some complementation to improve the efficacy of the treatment and to decrease post-therapeutic complications, especially during doxycycline and melarsomine therapy. Although the AHS updated its recommendations in 2018 and in 2020 (Nelson et al. 2018, 2020), the earlier protocol was used, as this research was started in 2014.

In the present study, moxidectin was applied as part of the three-dose alternate melarsomine therapy. This macrocyclic lactone is safe and efficient as a preventive drug against microfilariae (Genchi et al. 2019). McCall et al. (2014) studied moxidectin spot-on in microfilaremic dogs experimentally infected with *D. immitis*, and they did not observe any anaphylactic (adverse) reactions. Bagi et al. (2017) applied moxidectin in 22 microfilaremic dogs without any anaphylactic reactions. No anaphylaxis occurred even in our microfilaremic dogs during the present study. However, we know anecdotally from veterinarians and owners that moxidectin might elicit adverse reactions occasionally. Fok et al. (2010) reported weakness, anorexia, and nausea in 2 dogs of their 44 dogs infected with *D. repens* and treated with moxidectin. As moxidectin-elicited anaphylaxis might occur sometimes, we suggest pretreating microfilaremic patients with prednisolone and clopidogrel for one week. In addition, we give glucocorticoids and an antihistamine on the 1st day of moxidectin application together with temporary hospitalization as described earlier for ivermectin (Bowman and Atkins 2009, Yoon et al. 2013, Ware 2014, Nelson et al. 2020). Microfilariae of *D. immitis* are present in different organs histologically and can cause vascular lesions and other alterations like pulmonary granulomatous lesions (Rawlings et al. 1982, Calvert and Losonsky 1985, Calvert and Rawlings 1985, Ceribasi and Simsek 2012). This is also an argument why we have pretreated our

microfilaremic patients with clopidogrel as a platelet aggregation inhibitor and with prednisolone during the first application of moxidectin, similarly to Yoon et al. (2013). Whether clopidogrel is useful in the post-melarsomine therapeutic period, needs further investigations (Ames and Atkins 2020). Yoon et al. (2013) applied the anticoagulant enoxaparin, which belongs to the class of low-molecular-weight heparins (LMWH). They applied this drug before the surgical retrieval of adult heartworms from the main pulmonary artery via catheterization to prevent thromboembolism. We administered a different LMWH i.e., dalteparin sodium for similar purposes after the first melarsomine injection. It remains a question whether the favorably low number of mild and temporary respiratory side effects in our cases are due to the combined post-melarsomine therapy with prednisolone and dalteparin.

An interesting finding which has not been reported previously is that the adult heartworms disappeared from the main pulmonary artery in 2 dogs 6 to 10 days after the first moxidectin application. In another dog of Stage 3, the adult HWs also disappeared from the right atrium, 10 days after cardiac treatment of right heart failure, and they possibly moved to the pulmonary arteries. Similar observations were reported by others (Tjostheim et al. 2019, Pariaut et al. 2020).

In addition to its effect against microfilariae and susceptible larvae, moxidectin together with doxycycline has – at least partial – adulticide effect when applying these two drugs without melarsomine. The discussion of this “slow-kill” protocol is beyond the scope of my thesis and the interested reader can refer to a recent review article of Jacobson and DiGangi (2021). In the cases of the present study, the adulticide effect of moxidectin might have been added to that of melarsomine, which can also be an explanation for the favorable (100%) recovery rate of our patients.

Doxycycline is used as part of the complex HWD treatment. The recommended dose (10 mg/kg BID) by the AHS is relatively high (Nelson et al. 2014, 2020) and gastrointestinal side effects can occur. Schulz et al. (2011) examined the side effects of doxycycline in 386 dogs, during the therapy of various infectious diseases. In these patients, coughing occurred in 18.3%, diarrhea in 7%, and anorexia in 2.5% of their dogs. These authors also examined blood biochemistry parameters, observing an increase of ALT activity in 39.4% and increase of ALP activity in 36.4% of their cases. We also observed elevation in the liver enzymes in a few cases, however, we did not perform routine laboratory examination during the follow-up of all patients. In our study, we did not observe coughing as a side effect of doxycycline. The occurrence rate of gastrointestinal side effects in our patients (6.8%) was lower than in the report of Maxwell et

al. (2014). Doxycycline monohydrate has been reported to have less severe gastrointestinal complications than doxycycline hyclate (Greene and Calpin 2012). We applied the latter drug in the present study, whilst Maxwell et al. (2014) did not indicate which type of doxycycline they used. Our favorable results might also be due to the supplementation of probiotics during doxycycline application. There are experimental and human clinical studies on doxycycline therapy complemented by probiotics with a good outcome (Shah et al. 2013, Garrido-Messa et al. 2015). I did not find similar information in canine medicine. In 3/44 of our patients, it was necessary to decrease the 10 mg/kg BID dose of doxycycline to 5 mg/kg BID, similarly to the report of Maxwell et al. (2014). Additional medications (gastric protectants, antiemetics) were also applied to treat the gastrointestinal side effects in these dogs. Savadelis et al. (2018) reported that none of the dogs that received doxycycline 10 mg/kg for 28 days BID had Wolbachia DNA, but it was present in 25% of those who received 5 mg/kg BID. However, they recommended to apply the 10 mg/kg BID dosage for canine heartworm treatment and reduce it to 5 mg/kg BID in cases of severe gastrointestinal side effects. Carreton et al. (2020) studied recombinant *Wolbachia* surface protein (rWSP) in HW infected dogs, using three different doses of doxycycline during the AHS treatment protocol. Significant decreases of anti-rWSP antibodies were found after the administration of the three different doses of doxycycline evaluated. Based on these results, they raised the possibility that a lower dose than currently recommended would be sufficient to significantly decrease the *Wolbachia* population. During doxycycline therapy, follow-up monitoring of liver enzyme activities can be suggested, especially in older dogs and in patients with previous history of even subclinical hepatic disorders based on the study of Schulz et al. (2011).

Nelson et al. (2017) published a respiratory complication rate of 6.5% and 0% heartworm-related death utilizing the AHS protocol, including doxycycline, ivermectin, and the three-dose melarsomine therapy. These data are more favorable compared to those of the clinical field trials during the melarsomine approval process - without doxycycline and melarsomine - i.e., 22% and 7.0%, respectively (Keister et al. 1995). Maxwell et al. (2014) reported on the side effects of the three-dose alternate melarsomine therapy including ivermectin and regarding post-therapeutic complications and outcome. Altogether 7 (14%) of their dogs died, because of thromboembolism and progressive heart failure. One of those patients belonged to Stage 2, and two of them to Stage 3, whilst four dogs had caval syndrome, and were therefore categorized as Stage 4

In the present study, the number of dogs with local side effects were higher than that of Maxwell et al. (2014). However, the great majority of our patients showed only mild and temporary local reactions without clinical relevance. We applied post-therapeutic follow-up ultrasonography of the paralumbar muscles, which diagnostic procedure may also have increased the number of the detected cases with mild local alterations. In the two cases with severe, painful, apple-sized swelling we suspected individual local sensitivity to melarsomine as the injection procedure was the same as in the other dogs. Abscess formation did not occur in our patients, contrary to the three cases of Bagi et al. (2017). Maxwell et al. (2014) did not report on this complication. Incorrectly injected melarsomine can even cause spinal cord paralysis (Hettlich et al. 2003, Moore et al. 2013) which should be avoided with proper restraint and injection technique. Our favorable results regarding only mild and mainly temporary local adverse reactions can be due to the exact ultrasonographic determination of the injection site of melarsomine (Vörös et al. 2022a). Sedation with butorphanol and keeping the needle in site for 5 minutes to avoid leakage also proved to be useful. Namely, melarsomine is absorbed quickly from the musculature (Boehringer Ingelheim Immiticide® 2020, Zoetis Inc., Diroban® 2020, Vörös et al. 2022a). There are different opinions in the literature regarding the necessity of restriction and sedation of patients during melarsomine injection. This procedure is suggested even without sedation and having the dogs in standing position (AHS speaks out 2021) or to use analgesics like tramadol or hydrocodone (Ames and Atkins 2020, Nelson et al. 2020). Maxwell et al. (2014) mentioned the application of butorphanol as the part of adjunctive medication without any further details. Bagi et al. (2017) used general anesthesia achieved with a combination of intravenously applied xylazine, diazepam and ketamine. We chose butorphanol, a synthetic opioid drug which has both sedative and analgesic effects with minimal cardiovascular sequelae (Vin Veterinary Drug Handbook 2021).

Systemic side effects of melarsomine occurred at a very low rate in our study. Anorexia and diarrhea could have been caused by melarsomine (Boehringer Ingelheim Immiticide® 2020, Zoetis Inc., Diroban® 2020). Only mild and temporary coughing occurred sporadically in a few patients during the post-melarsomine period. This might have been due to minor thromboembolism elicited by the dying and decaying parasites or as side effects of melarsomine itself (Hoch and Strickland 2008b, Bowman and Atkins 2009).

Unlike in the study of Maxwell et al. (2014), none of our patients died, and all of them were treated successfully. However, it is difficult to compare the results of different studies due to the variation in the population involved in each study. For example, Maxwell et al. (2014)

reported cases from the lower Mississippi River Valley, one of the highest endemic areas in the USA. Although both their and our cases were treated at a university hospital, tending to attract more severely affected dogs, our patients were referred from variously infected parts of Hungary. Nelson et al. (2017) reported on a similar cohort to our dogs, and no death occurred among their patients when they applied the AHS protocol involving ivermectin.

In general, even our Stage 1 dogs did better after HWD treatment according to the owners' reports. This observation was prominent especially regarding the performance of working dogs. Among the 44 patients, 7 dogs belonged to Stage 3 with severe HWD, and they healed as well. These favorable results might be due to the AHS therapeutic protocol supplemented with the described, additional medications with special regard to moxidectin and to the applied, additional therapeutic measures.

HWD cases belonging into Clinical stages 3 and 4 can have severe sequelae of the disease which should be considered during the diagnostic work-up and therapy (Ames and Atkins 2020, Ware and Ward 2020, Becker et al. 2022b). These complications include pulmonary hypertension (PHT), right sided congestive heart failure (RCHF), and pulmonary thromboembolism (PTE) which can occur even together. The clinical symptoms and radiological changes of these disorders are less specific and can be even overlapped. Additional diagnostic procedures should be applied e.g., echocardiography for PHT and RCHF as well as D-dimer measurement, thrombocyte count determination, and computer tomographic angiography (CTA) for PTE **(Figure 10, 11, 12)** to achieve the exact diagnosis as described in more details in the review of Becker et al. (2022b). To diagnose PTE, the most versatile method is CTA which has been firstly published in Hungary by our research group (Becker et al. 2022b).

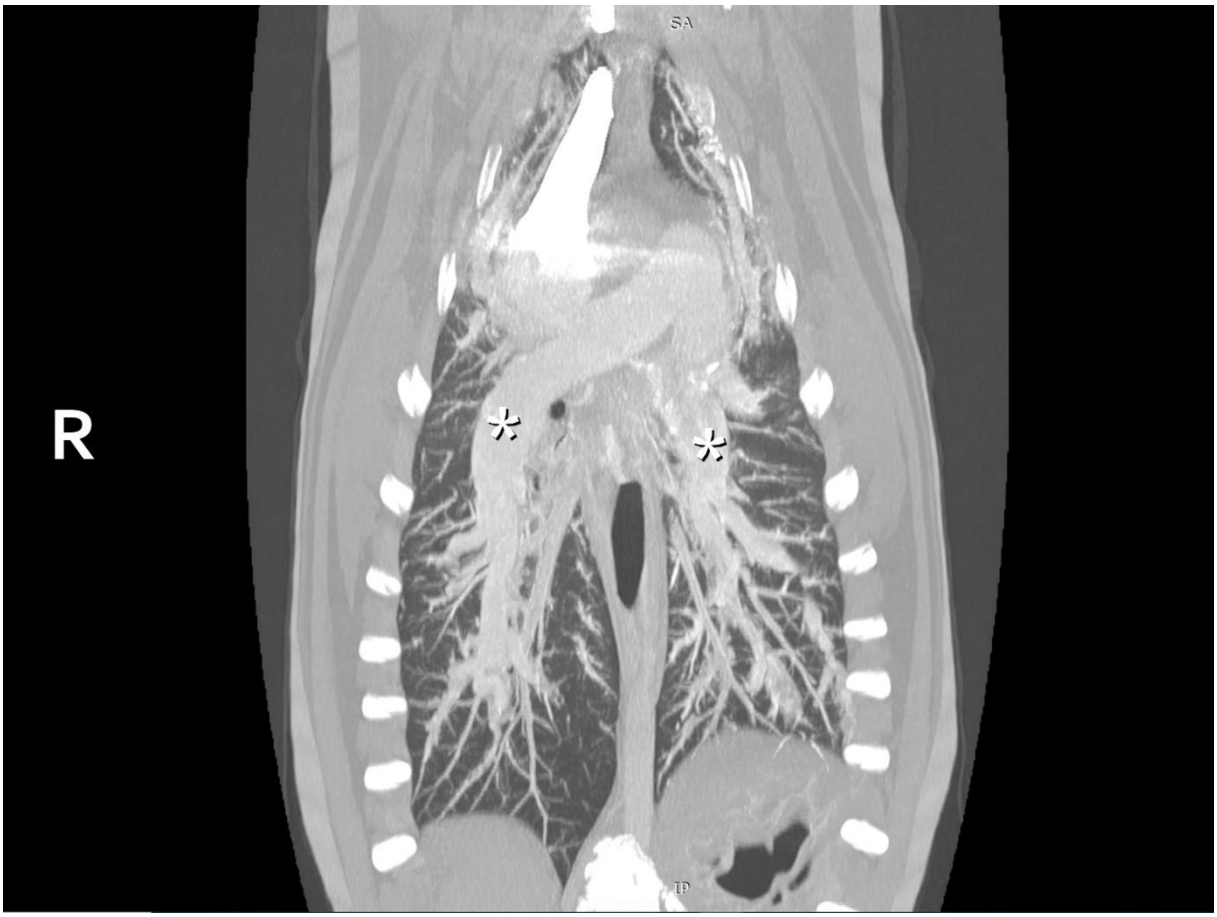


Figure 10. CT angiography of the chest of a dog with severe (Stage 3) heartworm disease. Dorsal plane oblique MIP (maximum intensity projection) reconstruction image. Caudal lobar pulmonary arteries (*) are tortuous and dilated. From Becker et al. 2022b. (With permission.)

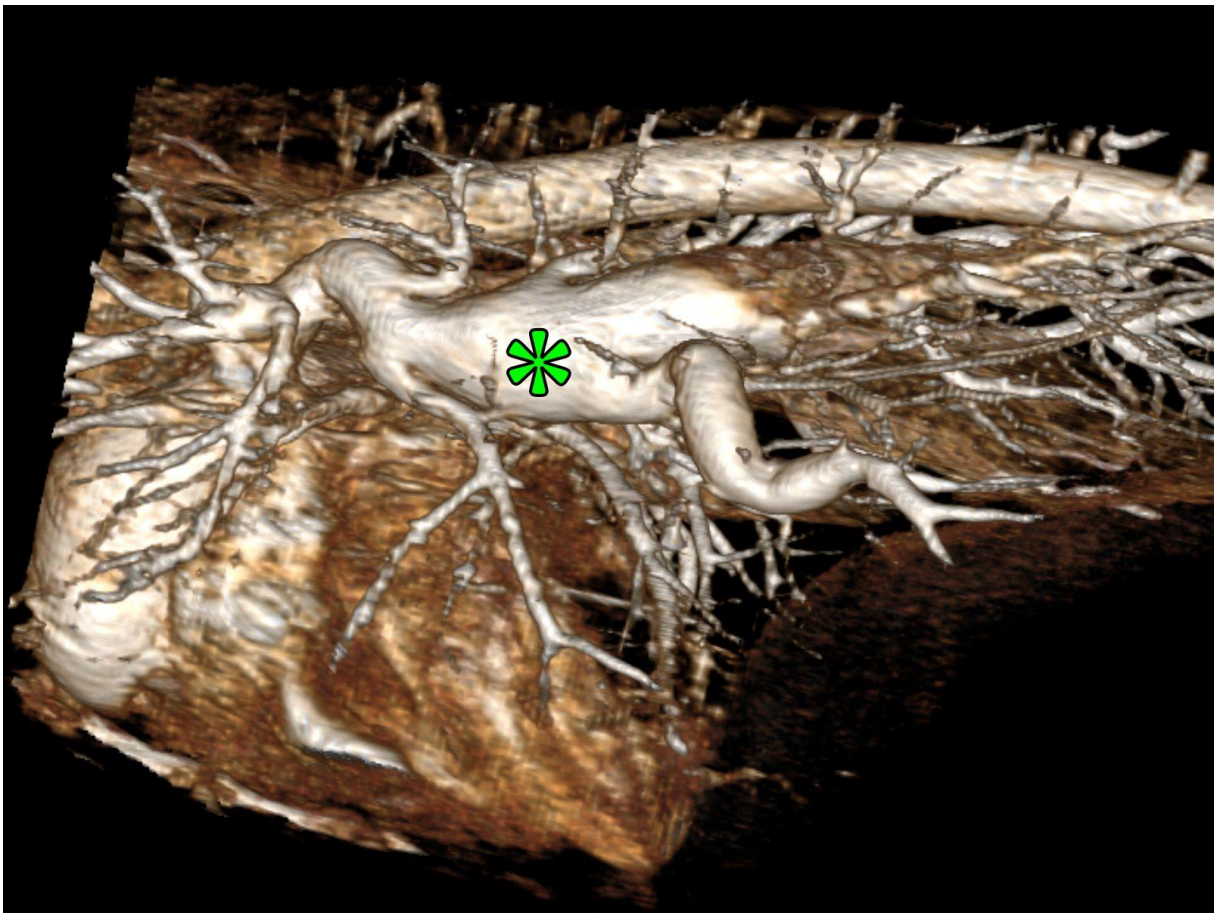


Figure 11. Severe (Stage 3) heartworm disease in a dog. CT angiography, 3D reconstruction of the pulmonary vasculature. The left caudal lobar artery (*) is extremely dilated and tortuous. Some other dilated pulmonary arteries can also be seen. From Becker et al. 2022b. (With permission.)

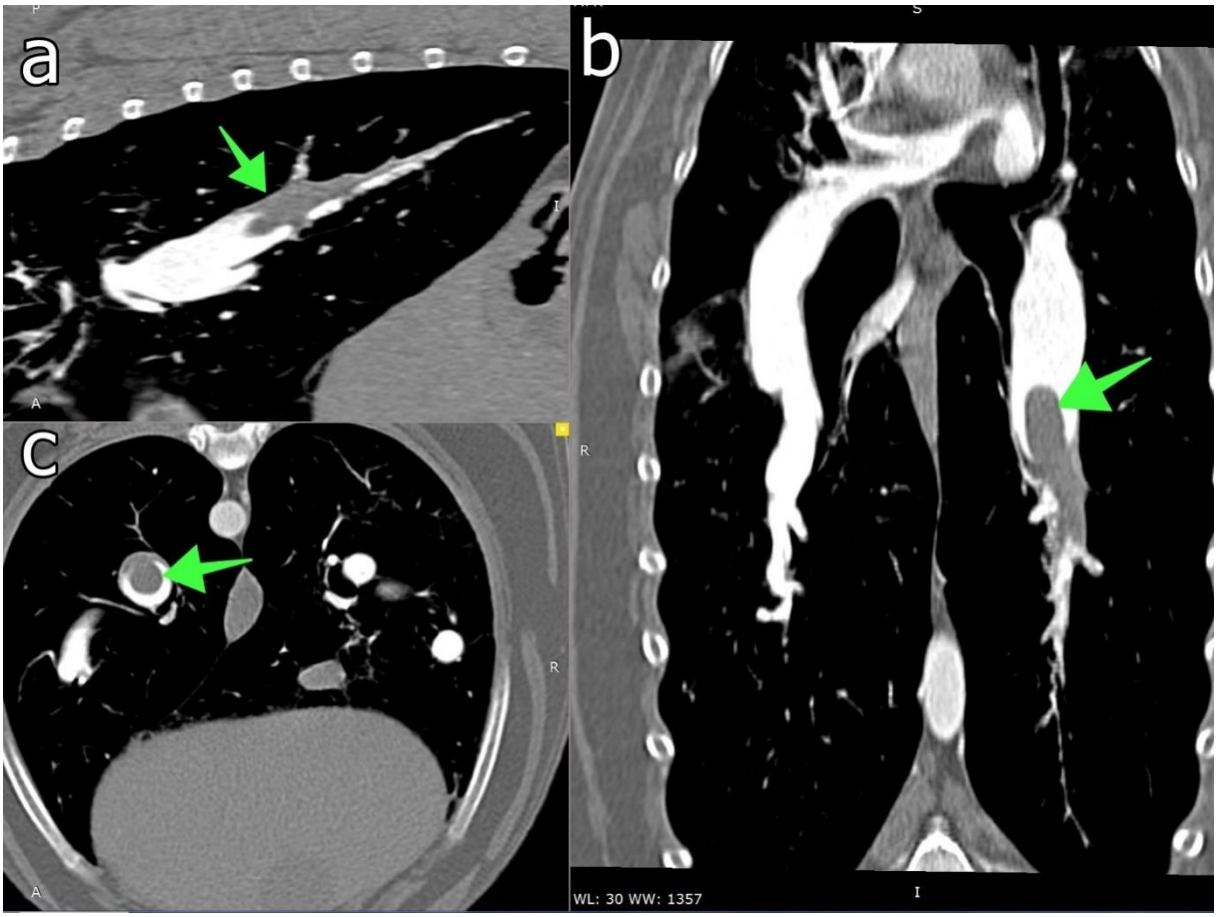


Figure 12. Pulmonary thromboembolism in a dog with severe (Stage 3) heartworm disease. CT angiography, sagittal (a), dorsal (b), and transverse (c) reconstructed images. Both caudal lobar arteries are dilated and tortuous. A large thrombus is visible in the left caudal lobar artery (arrows). From Becker et al. 2022b. (With permission.)

Less common complications are eosinophilic pneumonitis, eosinophilic granulomatosis, and pneumothorax. We did not observe these phenomena among our cases till now. As such, we do not have personal experiences on these complications. A literature review can be found in a recent publication of our research group (Becker et al. 2022b). As to the literature, preceding therapy of the aforementioned complications enable the specific treatment of HWD even in severely affected patients. Thereby, adequate satisfactory clinical and complete parasitological recovery can be reached in the majority of these dogs. They can have a good quality of life, although their exercise capacity might be limited to an everyday regular activity without more intensive workload. In some cases, PHT, RCHF and PTE might need a more or less long-time maintenance therapy.

The detailed clinical information on vena cava syndrome (Stage 4) is also beyond the scope of this thesis. Comprehensive description of this type of HWD can be found in the review of Farkas

and Vörös (2015), as well as in standard textbooks (Ware 2014, Vörös 2019, Ware and Ward 2020).

The present study has some limitations. The results are not supported by gross pathology and histopathologic examinations, as all patients recovered. Posttreatment observations were mainly done by the owners and/or were based on our physical examinations, on phone calls and email correspondence as described in the Materials and methods section. This type of follow-up is different from the written survey of Maxwell et al. (2014) and somewhat similar to the method of Nelson et al. (2017) who utilized phone calls to ask the questions. No regular follow-up studies of our patients were performed by radiographic and clinicopathological examinations. We applied our modified therapeutic protocol on all dogs. As such, no control group receiving only the original AHS schema was formed. The reason for this that our preliminary experiences were quite favorable. As such, we did not want to curtail our patients of the advantages provided by our modifications during the treatment of HWD.

Conclusions

In this research, during the treatment of heartworm disease, moxidectin was used instead of ivermectin, probiotics were added to the admission of doxycycline and the patients were sedated with butorphanol before the application of melarsomine injection. The exact place of the needle was also determined, by the help of ultrasonography. With the applied therapeutic modifications and complementary measures compared to the AHS 2014 protocol, satisfactory results were achieved regarding the outcome and the mainly mild post-therapeutic systemic and local complications with respect to the previous report of Maxwell et al. (2014). The mild post-therapeutic systemic alterations were basically not different from the satisfactory data of Nelson et al. (2017). All 44 patients were symptomless one month after the 3rd melarsomine injection and healed parasitologically at the control examination on the 271st day regarding the 33/44 dogs examined with Ag tests. Further research including regular follow-up by radiography and clinicopathological examinations might be useful to expand the results of this study.

3. Description the diagnosis and treatment of a dog with occult heartworm disease[3]

Introduction

In the case occult dirofilariosis (more precisely occult HW infection), there are living, adult heartworms in the infected animal, however, cannot be detected in the peripheral blood (Calvert et al. 1999, Nelson et al. 2014, Thomason and Calvert 2015, Atkins 2017, Ware and Ward 2020). This type of HW infection can have several reasons as described in the 1st Chapter of this thesis. Briefly: too low number in the peripheral blood to detect by the Knott test; previous macrocyclic lactone application for prevention of dirofilariosis; unisex infections with only male or female mf transmitted by the mosquito; or mf are eliminated from the peripheral blood by the immune system of the infected dog. The latter phenomenon is called true occult dirofilariosis (Wong et al. 1973, Rawlings et al. 1982). The Knott test and most commonly also the PCR assay reveals negative results, as there is no (not enough) circulating mf. During HWD, echocardiography can be of diagnostic value when adult HWs are seen in the main pulmonary artery and/or in the right heart (Lombard and Ackerman 1984, Brown and Gaillot 2008, Túri and Hetyey 2014, Bagi et al. 2017). The diagnostic method proved to be valuable also in one previous report including occult dirofilariosis cases as well (Badertscher et al. 1988).

The major goal of this case report is to demonstrate the main clinical features as well as the diagnostic and therapeutic procedures of a dog with true occult dirofilariosis which has not been published previously in Hungary. This case report is also an example of the diagnostic work-up and therapeutic management of severe HWD caused by occult dirofilariosis with special regard for applying multiplex Ag tests in these cases. The latter has never been published as to our search through the international literature.

History

A 4-year-old, 38 kg, American Staffordshire terrier male dog was referred to the clinic of University of Veterinary Medicine Budapest in 2017 with an anamnesis of a half year long coughing. A few weeks before admission, the dog received amoxicillin-clavulanic acid, azithromycin and ambroxol for a week. However, its general status did not improve, and the coughing became more severe. Before presentation, the dog also received furosemide for 2 weeks. The owners experienced, that the dog gradually became weaker and anorexic, lost

[3]This chapter is based on Vörös, K., Becker, Zs., Arany-Tóth, A., Gyurkovszky, M. and Farkas, R (2017): Okkult *Dirofilaria immitis* szívférgesség kutyában. Esetismertetés és irodalmi áttekintés. Magy. Állatorvosok Lapja. **139,** 675-685.

weight, had dyspnea, abdominal distension and fainting during the last few weeks. The patient received rabies vaccine four months and combinate vaccine against infectious diseases (distemper, leptospirosis, hepatitis) one year earlier. At the time of vaccination, it was dewormed with a combinate tablet of fenbendazole, pyrantel-pamoate and praziquantel. The dog was kept at Jászfényszaru, in a garden. Mosquitoes were common at the environment where the dog lived from its puppyhood never leaving this area. Neither repellents against mosquitoes nor macrocyclic lactones to prevent *Dirofilaria* infection were given before the referral to our clinic.

Physical examination

During the clinical examination, the patient was lethargic and reluctant to move. A mixed type dyspnea and abdominal distension were obvious. The developmental stage of the patient was appropriate regarding its breed, age, and sex. However, the dog was moderately emaciated. Rectal temperature was 38.6 °C, the pulse rate yielded 160/min, and the respiratory rate 40/min, respectively. During the detailed physical examination of the organs, its haircoat was dry and dull, and the elasticity of its skin was decreased. The mucosal membranes were cyanotic; however, the capillary refill time was normal (<2 sec). We did not observe nasal discharge. It was easy to provoke coughing. The cough was strong, long-lasting, repeated, dry, deep, and moderately painful. Stridor above the pharynx and the trachea was heard. Moderate, mixed type dyspnea was observed, mainly as a thoracic respiratory type. Bronchial sounds were heard diffusely above both sides of the thorax. In addition, crackles (crepitation) were evident, above the dorsocaudal part, mainly during inspiration. The percussion sound of the chest was duller and shorter than normal, especially at the dorsocaudal region. Regarding the circulatory system, the heartbeat was weaker during the observation and palpation of the chest. The heart sounds were rapid, rhythmic, and weaker during auscultation. The 1st and 2nd heart sounds could have been distinguished, whilst additional heart sounds and murmur were not heard. The pulse at the femoral artery were rapid, rhythmic, and weaker. The jugular veins were distended, and a positive (systolic) fluctuation was visible. The abdomen was distended, pear shaped, with severe undulation. During percussion of the abdomen, the sounds at the upper third were tympanic, below that the percussion sounds were dull, divided by a horizontal line which changed its position by elevating the animal. These signs were suggestive of the presence of free abdominal fluid. Deep palpation of the abdomen cannot be performed due to its distension. There were no changes during the physical examination of other organs.

Diagnostic imaging and routine clinicopathological examinations

Respiratory radiography was performed with a high frequency radiography machine (7X SUPER HF 650, PerkinElmer XRpad 4336 MED) in right laterolateral and dorsoventral projection. Right-heart enlargement, bulging of the main pulmonal trunk as well as dilatation of the pulmonary arteries and an interstitial-alveolar pattern of the caudal lung lobes were seen.

M-mode, two-dimensional, color Doppler and spectral Doppler examinations with an Esaote Mylab Gold 40 ultrasound machine reveled dilation of the right ventricle and right atrium as well as severe tricuspid and moderate pulmonary valve insufficiency was observed. Pulmonary hypertension was also stated based on the quantification of these valvular insufficiencies (vmax of regurgitation 4.5 m/sec, PG 81 mmHg and vmax 0.7 m/sec, PG 2 mmHg, respectively) based on Brown and Gaillot (2008). The diameters of the left ventricle in diastole and that of the left atrium in systole and diastole were below the normal limits suggesting low cardiac output of the left heart. The main trunk and the main left and right branch of the pulmonary artery were dilatated, and a few cross sections of adult heartworms were visible. During ECG-examination, sinus rhythm, as well as signs of enlargement of the right atrium and ventricle were observed. On abdominal ultrasonography, enlarged (congested) liver, dilatated hepatic veins and severe free abdominal fluid accumulation were visible.

Hematologic and routine serum biochemistry examinations performed at the Department of Pathophysiology and Oncology, UVMB resulted in the following clinicopathological alterations: leukocytosis, eosinophilia, moderate hyperglycemia, and mild hypomagnesemia.

Parasitological laboratory examinations

As to the history (the dog living in an endemic region) and the clinical signs, severe (Stage 3) HWD was suspected (among other diseases with right-heart failure, and ascites). The modified Knott test to detect mf was performed at the Department of Pathophysiology and Oncology, UVMB and it revealed a negative result. To control this finding, the Knott test was repeated at the Department of Parasitology and Zoology, UVMB from the same blood sample, and its result was also negative. Nevertheless, PCR was also performed at the Department of Parasitology and Zoology, UVMB as described in Chapter 1. Neither *D. immitis* nor *D. repens* infections were found.

Based on parasitological results as well as on the presence of adult HWs in the main pulmonary artery observed with echocardiography, occult dirofilariosis was diagnosed clinically. In order to demonstrate the presence of adult female worms, the following four antigen tests were used

with the same blood sample: Idexx SNAP Heartworm RT Test (Idexx Deutschland); VetScan VS2 HW test (Abaxis, USA); Witness Dirofilaria (Zoetis, USA); DiroCHEK Canine Heartworm Antigen Test Kit (Symbiotic Co. USA). Antigens of *D. immitis* were detected with each of these tests.

Diagnosis of occult dirofilariosis was established, supported by the aforementioned echocardiographic and parasitological findings. As the dog never received macrocyclic lactones before, true occult HWD was stated as discussed in Chapter one of this thesis. The patient was categorized into Clinical Stage 3 based on the physical examination and radiographic findings and confirmed by echocardiography.

Treatment

The severe congestive right-sided heart failure was treated with furosemide (1.6 mg/kg BID), potassium- and magnesium-aspartate (1.8 mg/kg SID), enalapril (0.4 mg/kg SID), and pimobendane (0.2 mg/kg BID). The clinical signs improved in a week: the patient's appetite become better, ascites decreased, coughing has ceased, and fainting occurred only once in the next few weeks.

The complex therapy of HWD as to the AHS recommendation (Nelson et al. 2014) was started on the 21st day after the clinical signs of congestive heart failure remarkably improved. The dog was treated with moxidectin 2.5%/imidacloprid 10% (Advocate® spot-on, Bayer Animal Health GmbH, Germany) to eliminate L3 and L4 larvae, four times monthly. In addition, doxycycline was started in a dose of 10 mg/kg BID per os, against the endosymbiont *Wolbachia pipientis* for 28 days. The adulticide melarsomine (Immiticide) injection was administered intramuscularly, the first injection was given at the 80th day, instead on the 60th day (suggested by Nelson et al. 2014) because of the previously discussed cardiorespiratory sequalae of the severe HWD.

No side effects of any applied drugs were observed during the complex therapy. The general status of the dog showed further improvement, the patient was in a good condition 6 months after the beginning of the complex therapy. The radiographic and echocardiographic alterations improved. The repeated Knott test and Ag tests revealed negative results at that time. The patient received the same medications for chronic treatment of the cardiac failure, which did not deteriorate even during mild to moderate activity. The dose of furosemide was decreased to the third of the initial dose, and the potassium- and magnesium aspartate were also decreased to every other day (EOD) application. The patient was in a good general state 11 months after

the first examination. The radiographic and echocardiographic alterations diminished even further.

Discussion

The diagnosis and treatment of HWD are detailed in the previous chapters of my thesis. As the Knott-test is negative in occult dirofilariosis, just as the PCR technique, this form of HWD can be diagnosed by applying more antigen tests of different manufacturers with the same blood sample to detect adult, female HWs (Becker et al. 2022a). In the present case, HW antigen tests from 4 different manufacturers were used, and all of them were positive. Another proof of HWD was, that adult HWs were seen in the main pulmonary artery similarly as reported by Badertscher et al. (1988). In the past, before the use antigen-tests, the diagnosis of occult heartworm disease was made by selective angiography, indirectly visualizing heartworms (Wong et al. 1973, Matic and Herrtage 1987). In the literature, the use of detecting antibodies against microfilariae (e.g., with immunofluorescent antibody procedure) has been described only for scientific research purposes (Wong et al. 1973, Rawlings et al. 1982, Boto et al. 1984). This might be useful for diagnosing the disease in a clinical setting. However, I have found one preliminary study where two versions of feline HW antibody test have been recently used in canine HWD after preheat treatment, the latter to disrupt antibody-antigen complexes (Lane 2021). In that study, 14/100 stray adult dogs had positive results with feline HW Ag test. Nevertheless, the applied feline antibody tests are not commercially validated/approved for dogs.

In conclusion, occult HW infection can arise diagnostic dilemmas as the Knott test and the PCR will reveal negative results. In these cases, positivity of more Ag tests can support the diagnosis of HWD, even if no adult HWs can be seen within the main pulmonary artery during echocardiography (Becker et al. 2022a, Vörös et al. 2022b). Briefly, the chance for a cross reaction with other parasites is less, if more Ag tests provide positive results for HWD as outlined in Chapter 1 and 2 of my thesis. In questionable cases, the patient could receive macrocyclic lactone monthly, just as in prevention, supplemented with doxycycline in the first 28 days (Atkins 2017). The macrocyclic lactone therapy, namely ivermectin and moxidectin is also efficient against *A. vasorum* and *S. lupi*, those can give cross-reaction with *D. immitis* (Le Sueur et al. 2010, Jacsó 2014, Borgeat et al. 2015).

The present chapter of my thesis is based on our article of Vörös et al. (2017) which is the first report on true occult dirofilariosis in Hungary. This case report is also an example of the diagnostic work-up and therapeutic management of severe HWD during occult dirofilariosis.

New scientific results of the thesis

- Where concomitant infections by *D. immitis* and *D. repens* occur, the sensitivity of the Vetscan Ag test was similar, and its specificity was lower compared to other studies carried out in the USA where only *D. immitis* occurs.
- In cases of positive *D. repens* and negative *D. immitis* PCR results, occult dirofilariosis can be responsible for the positive *D. immitis* Ag tests as well as for their lower specificity compared to other studies.
- In the lack of microfilaraemia, like in occult dirofilariosis, multiple Ag tests from the same blood samples should be performed, to increase the diagnostic accuracy of HWD.
- As part of the complex treatment, moxidectin is not only effective against microfilariae as well as L3 and L4 stages but its partial adulticid effect can contribute to the effectivity of HW therapy.
- Gastrointestinal side effects of doxycycline therapy can be decreased by probiotics supplementation as an additive to the complex treatment of HWD.
- Sedation with butorphanol and ultrasonographic determination of the location of the injection needle can increase the safety of the melarsomine injection and decrease the severity of its local side effects.
- Occult heartworm disease was firstly described in Hungary.

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