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STUDIES ON THE EFFICACY OF SOME MARKETED *DIROFILARIA IMMITIS* ANTIGEN TESTS IN DOGS

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2015

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1. INTRODUCTION

Canine heartworm disease caused by *Dirofilaria immitis* is a widespread and emerging parasitosis of dogs in Europe and in other parts of the world. In Hungary the first autochthonous infection was reported in 2009. Since that time the number of infected animals has been increasing in many parts of the country. The reliable diagnosis is very important to the veterinary community and pet owners, because the parasite can cause severe cardiopulmonary disease, occasionally even with fatal outcome.

The accurate diagnosis is especially essential in Hungary as well as those countries, where the other *Dirofilaria* species, e.g. *Dirofilaria repens*, is also present, because it is not easy to diagnose in practice whether one or both *Dirofilaria* species causes the infection of dogs.

These kinds of serological tests are easy to perform, practical and quick. Another benefit to the antigen tests is that the occult infections can also be detected with them.

2. LITERATURE REVIEW

2.1 BIOLOGY OF *DIROFILARIA IMMITIS*

2.1.1 Taxonomy and morphology

Dirofilaria immitis, commonly known as canine heartworm, belongs to the Family Onchocercidae, within the phylum Nematoda (Klotschko and Wallace, 2010). Since heartworm was first identified in 1856 by Dr. Leidy, numerous species have been discovered (Boreham and Atwell, 1988). The genus *Dirofilaria* consists of 27 apparently valid species and 15 species of questionable validity (Dantas-Torres and Otranto, 2013).

Nematodes of the genus *Dirofilaria* are elongated and thin with round anterior extremity and rudimentary buccal capsule without lips and small cephalic papillae. Adult heartworms vary in length. Adult females of *Dirofilaria immitis* are approximately 25-30 cm, males are shorter, about 10-15 cm long in their natural hosts (Simón et al., 2012). Both males and females have a diameter of approximately 1 mm (AHS, 2014).

2.1.2 Life cycle

The life cycle of *D. immitis* is indirect (Figure 1).

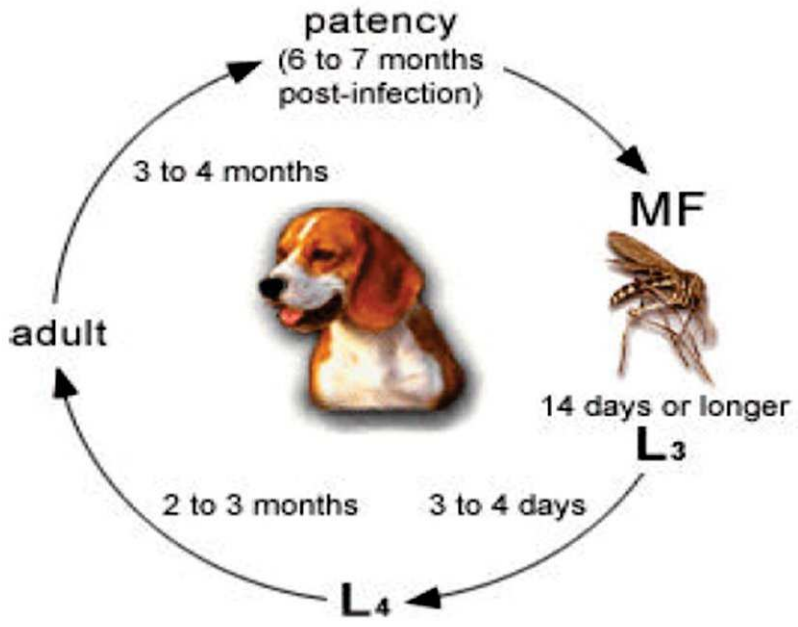
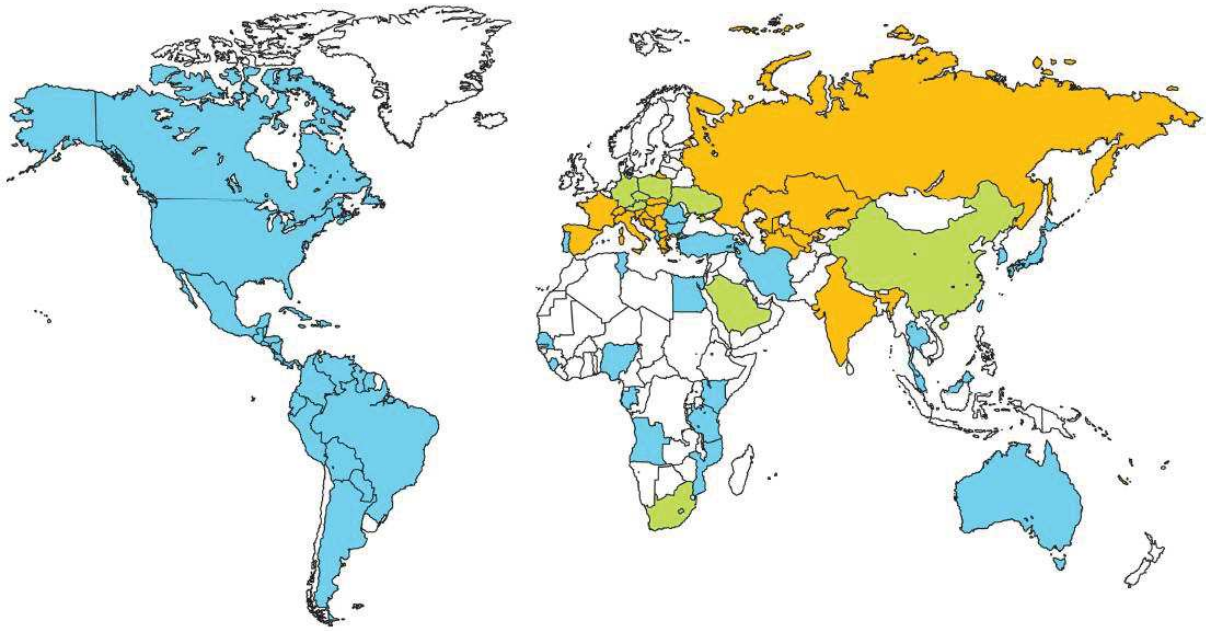


Figure 1: The life cycle of *Dirofilaria immitis* (Kotani and Powers, 1982**)**



During the last 14 years a rapid expansion of the parasitosis has been observed in central and northern countries of Europe (Morchón et al., 2012). Hungary is currently considered as endemic country for heartworm (Fok, 2007).

The reasons believed for this northern spread is an increase in the movement of dogs from endemic states to nonendemic states and a decrease in the use of pesticides to control mosquito populations because of their effects on the environment and animals (Selby et al., 1980; Genchi et al., 2011). Another possible cause of spreading is the global warming, which influences the spreading of hematophagous arthropods acting as vectors of different parasitic infections, such as dirofilariosis (Genchi et al., 2010). The increasing temperature facilitates the reproduction of vectors and parasites by rising the number of days suitable for their development (Morchón et al., 2012). The change in distribution of heartworm on the European continent can be seen in Figure 3.

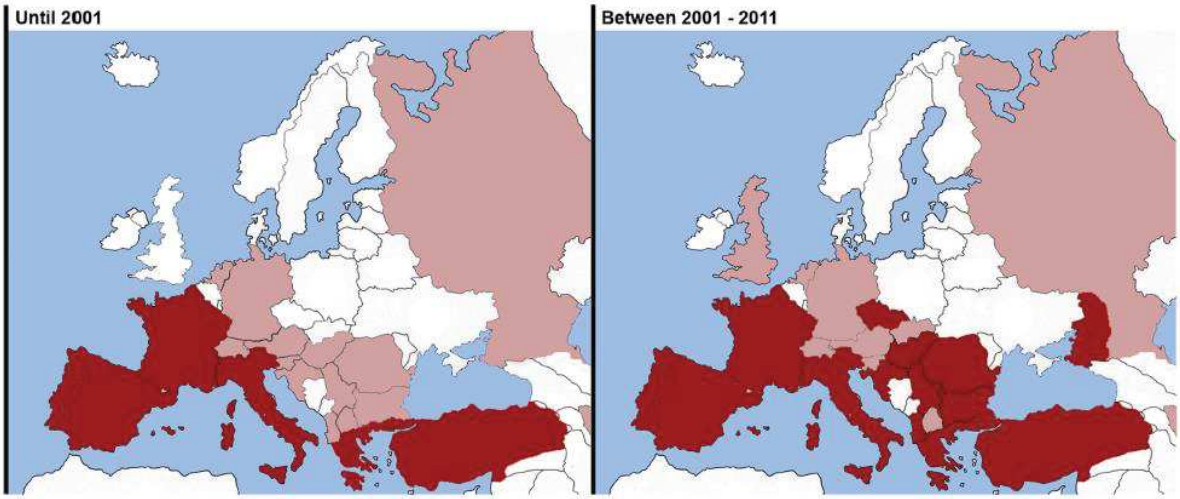


Figure 3: **Comparison of geographical distribution of heartworm disease in dogs in Europe. Endemic areas are red colour. Sporadic cases are pink colour (**Morchón et al., 2012**)**

aureus) was also reported (Tolnai et al., 2014). Mosquitoes collected in the southern parts of the country carried *D. immitis* at a higher rate then *D. repens* (Fok, 2007).

The presence of *D. repens* in Hungary has been first reported in a dog in 1998 (Széll et al., 1999). Nationwide studies were carried out in 2006 to investigate dirofilariosis of dogs caused by *D. repens*. The prevalence of this nematode infection was approximately 14%. Local prevalence under suitable environmental conditions (humid areas, presence of suitable carriers) was as high as 30% (Genchi et al., 2011). Recently, findings indicate that *D. repens* is present in all regions of country but unevenly distributed (Fok, 2007; Fok et al., 2007). Epidemiological survey conducted by Fok Eva and her co-workers in 2009 showed that most checked animals were positive (293/1610/18.2% of dogs and 3/67/4.5% of cats) were found on the watershed of the Danube and Tisza River (Fok, Jacsó, 2009).

2.3 DIAGNOSIS OF CANINE HEARTWORM INFECTION

The diagnosis of dirofilariosis poses problems in the countries where *D. immitis* and *D. repens* are present. It is very important to know the species that caused the infection for treating properly the infected dogs (McCall et al., 2008; Bowman and Atkins, 2009). The demand for simple, rapid and reliable techniques has increased considerably where both *Dirofilaria* species occur.

The first stage larvae, called microfilariae, of *D. immitis* can be observed under a light microscope in a drop of blood sample of infected dogs if there are many of them. This simple method is a quick, non-concentration test for detecting microfilariae. If there are only very few microfilariae in the sample they have to be concentrated with modified Knott test (Genchi et al., 2005) which was first described in 1939. When microfilariae are found, the species must be identified, since this test is not species specific (AHS, 2014). Knott test has been the gold standard diagnostic method for heartworm infection. Various filter techniques, including the millipore and nucleopore kit methods also give unreliable results, differentiating the microfilariae less accurately. Multiple sampling significantly increases the likelihood of a diagnosis (AHS, 2014).

In the occult heartworm infection, which occurs in 20% of the cases, no microfilariae are found in the dog's blood (AHS, 2014). Occult infections are dangerous because the dog will not receive appropriate care and thus may contribute to the further transmission of the parasite. Therefore, the Knott's technique is not recommended as a stand alone diagnostic test for *D. immitis* (AHS, 2014). Moreover, microfilariae have a general diurnal periodicity, being most numerous in the blood stream during the early morning and late evening (Rhee et al., 1998; Hawking, 1967). In temperate climates there is a distinct seasonal periodicity. The microfilaremia is reduced during the winter (Boreham and Atwell, 1988; Anderson, 1992). Thus, periodic fluctuations are likely to result in a false-negative diagnosis of heartworm infection (Boreham and Atwell, 1988). Again, the sensitivity of tests for microfilariae is not therefore considered sufficient to rule out the infection in case of a negative result.The other reasons for false negative results in using microfilaria concentration techniques also include inadequate sample size and the host's immunity prior to therapy (Anderson, 1992).

Species can also be defined by the histochemical staining of anatomical regions with phosphatase activity (Chalifoux and Hunt, 1971). *D. immitis* microfilariae harbour two phosphatase activity zones near the anal and excretory pores, whereas *D. repens* has only one near the anal pore. *Dipetalonema reconditum* stains over the entire microfilariae. Figure 4 shows *Dirofilaria immitis*, *D. reconditum*, *D. repens* with evidence of enzyme activity.

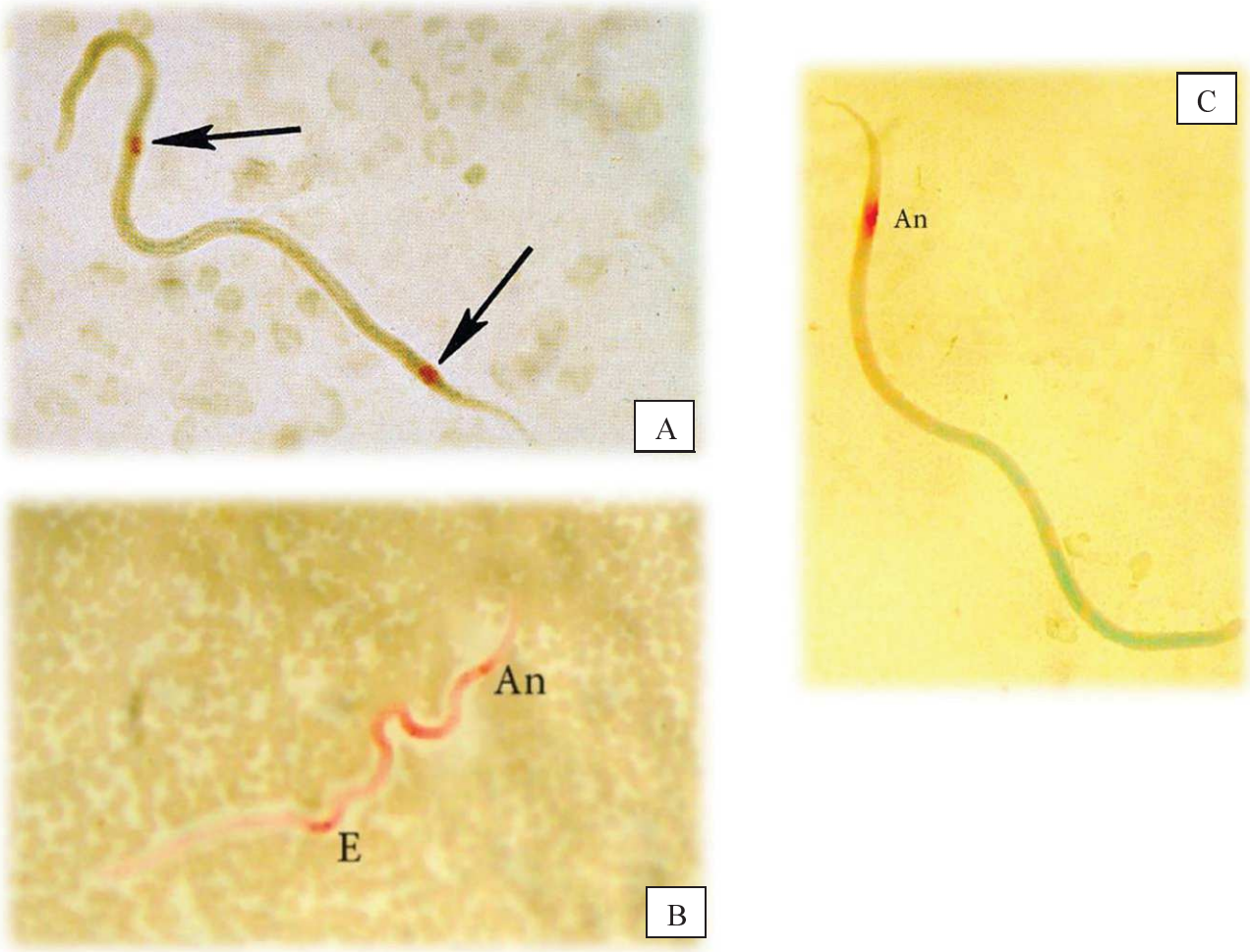


Figure 4: **A: *Dirofilaria immitis* with evidence of enzyme activity in areas of excretory pore and anal pore (arrows) B: *D. reconditum* with evidence of uniform enzyme activity throughout the entire body, especially between the excretory (E) and anal pores (An). C: *D. repens* microfilaria showing acid phosphatase activity at one anal pore (An)** (Chalifoux and Hunt, 1971; Ravindran, et al., 2014)

2.3.2 Immundiagnostics methods

Immunochromatographic strip tests have several disadvantages. The sensitivity of these tests is normally lower compared to other conventional techniques like PCR (Favia, et al, 1996; Ranjbar-Bahadori et al., 2007). They do not give quantitative results, there is decreased precision and limited sensitivity due to imprecise sample volumes, it can usually only test for one analyte, membrane pores can get blocked, and pre-treatment of non-fluid sample is necessary (Favia, et al, 1996; Trumpie et al., 2009). Problems can occur if only male worms are present in dogs (Atkins, 2003). If only a few female worms are mature and producing antigen then the levels in the blood may not be high enough to be detected by the test (Atkins, 2003). In addition, *D. immitis* antigen does not appear in the blood of infected dogs until 6 to 9 months after infection (Frank et al. 2001). Therefore, a positive test for heartworm often indicates an infection acquired sometime during the previous year.

In addition, further pan-filarial primers for the diagnosis of multiple infections have been recently designed; this permitted a single PCR reaction to differentiate between *D. immitis* and five other filariae found in dogs (Rishniw et al., 2006).

3. MATERIALS AND METHODS

3.1 ANIMALS, SAMPLE COLLECTION AND STORAGE

3.2.3 Serological studies

Based on the results of PCR the following sera were tested:

Group A: sera samples of 15 dogs infected with *Dirofilaria immitis*

Group B: sera samples of 15 dogs infected with *Dirofilaria repens*

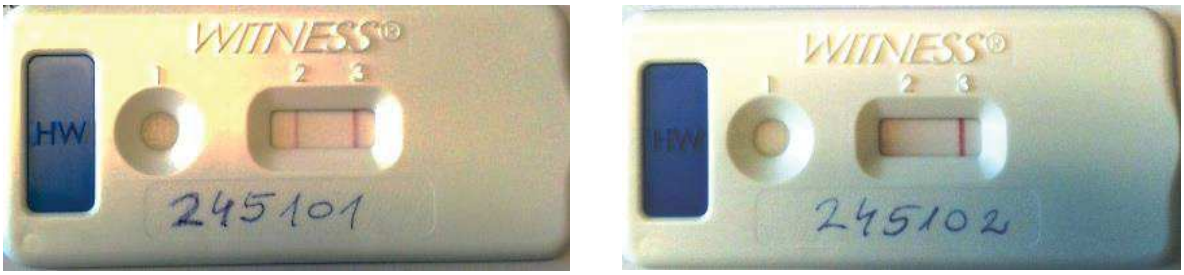
Group C: sera samples of 15 dogs infected with *Dirofilaria* immitis and *Dirofilaria repens*

Group D: sera samples of 15 dogs without *Dirofilaria* infection.

The following commercialized tests were evaluated:

WITNESS®DIROFILARIA (Zoetis Inc., Lyon Cedex, France)

This test is based on rapid immunomigration (RIM) technology. It is highly specific (100%), with sensitivity values ranging from 71% to 95% (Courtney, 2001; McCall et al., 2001). The test uses antibodies directed against specific epitopes of a soluble antigen of *D. immitis* in canine and feline whole blood, plasma or serum. The sample that contains this antigen is put into contact with sensitised gold particles. The resultant complex then migrates on the membrane before being caught in a reactive area, where complex concentration creates a strongly apparent pink-coloured band (Figure 5). A control band is located on the opposite side of the membrane to ensure that the test is performed properly.



A **B**

Figure 5: **Results of WITNESS®DIROFILARIA kit. A: Positive result: two vertical pink lines. B: Negative result: one vertical pink line**

FASTest® HW Antigen (Diagnostik MegaCor, Hörzbranz, Austria)

It is a lateral flow immunochromatographic test (sandwich system with 2 different antibodies, 1 of which is membrane-fixed and 1 bound to gold particles) for rapid detection of heartworm antigen in canine and feline serum, plasma or whole blood. It has 98.6% sensitivity and 99.1% specificity for the detection of *D. immitis* antigens stated in the instructions by the manifacturer. If *D. immitis* antigen is present in the sample, an immune-complex will be

formed. The serum specimens were allowed to thaw to laboratory ambient temperature (21–22°C). Briefly, two drops of migration buffer was added to 30 µL serum in a test casette and the cassete was placed on a flat surface. A control band is located on the opposite side of the membrane to ensure that the test is performed properly. The appearance of the S and C lines after a migration time of 15 minutes indicates a positive result. The appearance of the C line alone indicates a negative result. If the C line is not present, the test is considered invalid and is repeated. We evaluated all samples at 15 min (Figure 6).

A

B

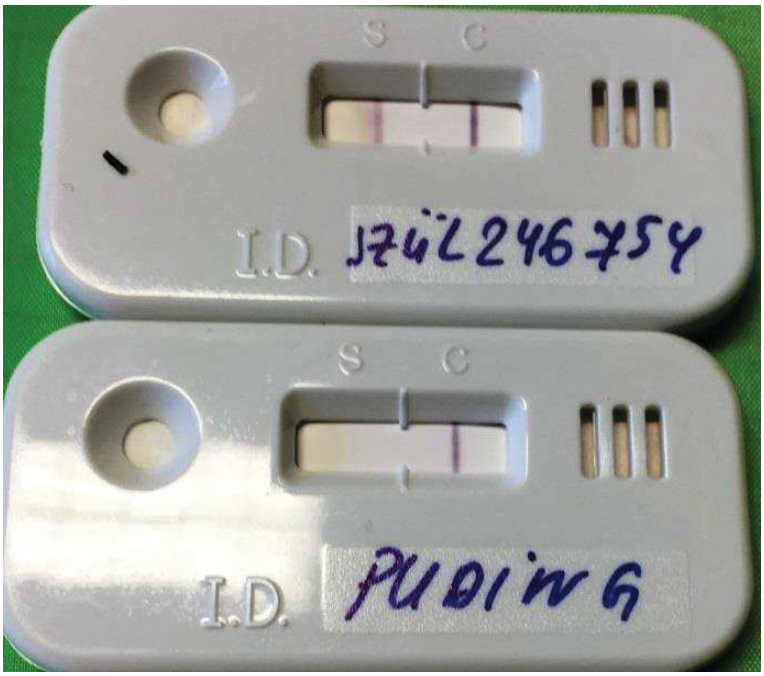


Figure 6: **Evaluation of FASTest® HW Antigen Test Kit: positive (A) and negative (B)**

DiroCHEK® (Synbiotics Corporation, San Diego, USA)

This is an ELISA test for the detection of adult *D. immitis* antigen in canine and feline plasma or serum. DiroCHEK® is highly specific (100%), its sensitivity ranges between 85% and 100%.(McCall et al. 2001;Gillis et al., 1984) Test results can be obtained within 15 minutes. The reaction wells are coated with antibodies directed against *D. immitis* antigen. Another antibody is labelled with horseradish peroxidase. Any antigen present in plasma or serum is bound by the antibody and coated with the enzyme-linked antibody to form a specific complex. Any free enzyme-linked antibody is washed away, and a chromogenic substrate is added. In the absence of *D. immitis* antigen, no colour change will be observed.

The development of a blue colour specifically indicates the presence of *D. immitis* antigen from heartworms (Figure 7).

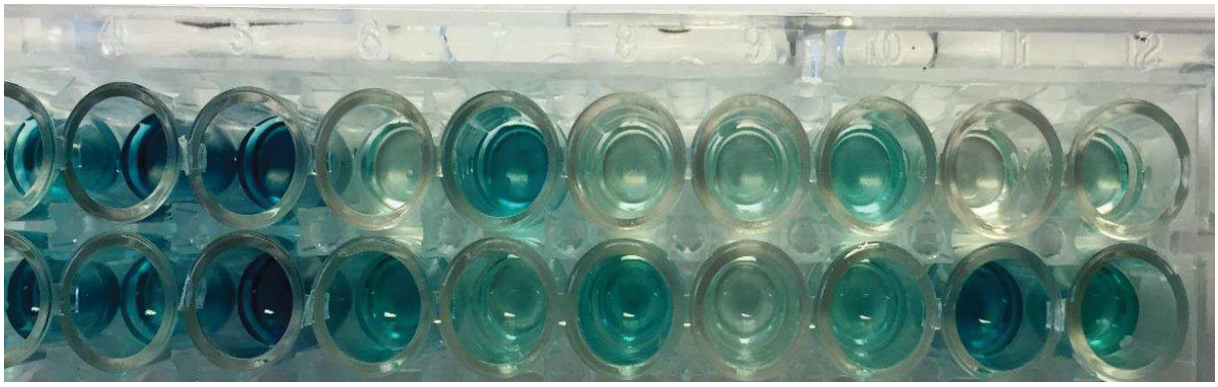


Figure 7: **Results of DiroCHEK® test based on colour reactions.**

Fassisi® CanDiro (Fassisi, Göttingen, Germany)

Fassisi® CanDiro strip is an immunosandwich assay for the rapid detection of heartworm antigen in canine serum, plasma or whole blood. It has 94.12% sensitivity and 99% specificity for the detection of *D. immitis* antigens, as stated by the manufacterer. Briefly, two drops of migration buffer was added to 50 µL serum in a sample well and the test strip result read within 10 min. The strip has two lines: a control line (C) (heartworm antigen – gold labeled antibody complexes) and a test line (T) (anti canine heatworm antigen-gold labeled antibody complexes). The appearance of the T and C lines after a migration time of 10 minutes (min) indicates a positive result. The appearance of the C line alone indicates a negative result. If the C line is not present, the test is considered invalid and is repeated. It is recommended that strips giving ambiguous (faint color at the T line) are considered nevertheless positive. We evaluated all samples within 10 min (Figure 8).

A

B

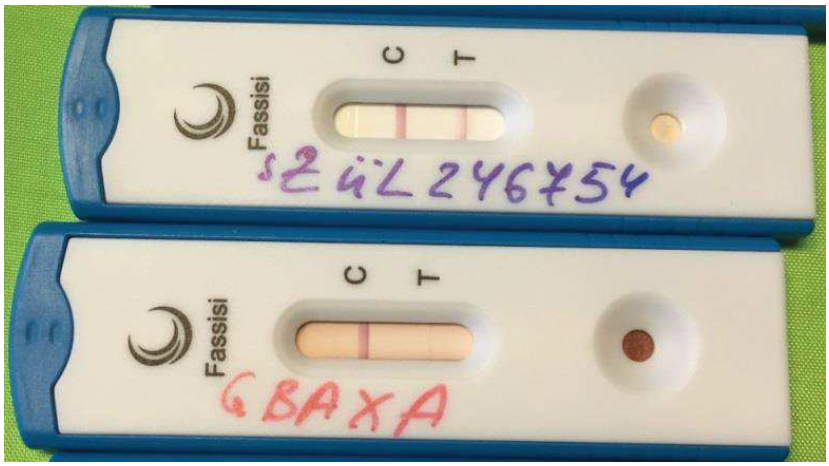


Figure 8: **Results of Fassisi® CanDiro test kit positive (A) and negative (B)**

3.3 DATA ANALYSIS

The derived data was tabulated in appropriate worksheets using the Microsoft Excel and evaluated.

The sensitivities, specificities, efficiency, accuracy, negative and positive predicted values were calculated as follows (Bland, 2000):

Sensitivity (%) = a/a+c x 100

Specificity (%) = d/d+b x 100

Efficiency (%) = a+d/a+b+c+d x 100

Negative Predicted Value (%) = d/d+c x100

Positive Predicted Value (%) = a/a+b x 100

Accuracy (%) = a+d/total number of samples x100

a = number of true positive

b = number of false positive

c = number of false negative

d = number of true negative

4. RESULTS

A total of 60 canine sera samples were used to evaluate four commercially available *D. immitis* antigen tests. The data of all tests have been summarised in Tables 1 and 2. All tests fulfilled the criteria for test validity. Interpretation was adopted by following the instruction of these tests for veterinary practitioners based on colour detection by naked eyes.

All of the sera samples of dogs having neither *D. immitis* nor *D. repens* infection in Group D were negative with each antigen tests (Table 1).

Three *D. immitis* antigen kits gave false positive reaction with one from 15 sera of Group B, each specificity was 93.3% (Table 1). DiroCHEK® had worse specificity (66.6%), with this kit, 5 out of 15 sera samples of dogs infected with *D. repens* gave false positive results (Table 1).

Groups	WITNESS® DIROFILARIA		Fassisi® CanDiro		*FAST*est® HW			
A (n=15)	12/15 (80.0) [a]	3/15	13/15 (86.7)[a]	2/15	11/15 (73.3) [a]	4/15	13/15 (86.7) [a]	2/15
B (n=15)	1/15	14/15 (93.3)[b]	1/15	14/15 (93.3) [b]	1/15	14/15 (93.3) [b]	5/15	10/15 (66.7) [b]
C (n=15)	11/15 (73.3) [a]	4/15	12/15 (80.0) [a]	3/15	10/15 (66.7) [a]	5/15	12/15 (80.0) [a]	3/15

If the results of the study with samples from group A and C are evaluated together, sensitivities of the WITNESS®DIROFILARIA, Fassisi® CanDiro and DiroCHEK® test kits are 76.7%, 83.3% and 83.3%, respectively (Table 2). In this case *FAST*est® HW Antigen has the lowest sensitivity (70.00 %) (Table 2).

According to this kind of calculation the specificity of three tests (WITNESS® DIROFILARIA Fassisi® CanDiro and *FAST*est® HW Antigen) is 96.7%, and it is 83.3% for DiroCHEK®. The accuracy of the four test kits ranges between 83.3 and 90% (Table 2). Positive predictive values (ie, probability of heartworm infection for a dog with a positive test result) were ranging 83.3%-90.0% for all 4 test kits (Table 2). Negative predictive values (ie, probability that a dog with a negative test result would be free from heartworm disease) were lower, 76.3% for the *FAST*est® HW Antigen test kit, 80.6% for the WITNESS®DIROFILARIA test kit, and 83.3% for the DiroCHEK® test kit. Negative predictive value of the Fassisi®CanDiro test kit was the higher (85.3%).

Table 2: Data obtained with 4 commercial heartworm antigen tests

	No. of samples								
	True positive	**True negative**	**False positive**	**False negative**	**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**	**Predictive Value (+)**	**Predictive Value (-)**
WITNESS® DIROFILARIA	23	29	1	7	76.7	96.7	86,7	95.8	80.6
Fassisi® CanDiro	25	29	1	5	83.3	96.7	90.0	96.2	85.3
*FAST*est® HW Antigen	21	29	1	9	70.0	96.7	83.3	95.5	76.3
DiroCHEK®	25	25	5	5	83.3	83.3	83.3	83.3	83.3

5. DISCUSSION

Dirofilaria immitis, the agent of canine heartworm disease, causes severe disorders and even death in dogs in many parts of the world (Boreham and Atwell, 1988). Antigen test kits, with which the circulating antigen of only female *D. immitis* can be detected are widely used in veterinary clinics, are highly sensitive and specific for heartworm infection in dogs (Atkins, 2003; McCall et al., 2008). However, the results depending on the commercially available in-clinic antigen assays. Based on the publications the microtiter plate ELISA has the highest sensitivity, followed by the membrane-based ELISA, and the lateral flow immunochromatographic test (Courtney and Zeng, 2001; Atkins, 2003).

The accurate antigen test is pivotal to early diagnosis and treatment of infected dogs in those cases when the animals may be amicrofilaremic due to unisex infection, or they may possess anti-microfilaria antibodies or macrolids used for chemoprophylaxis killed the microfilariae of *D. immitis* (AHS, 2014). In those countries where both *Dirofilaria* and other filarial species (e.g. *Dipetalonema reconditum*) are present (Weil et al., 1984; Morchón et al, 2012) it is especially important to diagnose the heartworm infection correctly in dogs with low heartworm burdens or having low levels of circulating heartworm antigen too in order to ensure prompt treatment (Atkins, 2003). Nowadays Hungarian veterinary surgeons also rely on rapid in-clinic antigen tests of *D. immitis* to screen for canine heartworm infection because besides the long lasting occurrence of *D. repens* in the country the parasitosis caused by *D. immitis* has been emerging in the local dogs (Fok and Jascó, 2009; Tolnai et al., 2014). From practical point of views it is impossible to figure out whether the infected dogs harbour microfilariae of *D. repens* and/or *D. immitis*. In these cases the detection of *D. immitis* antigen with fast in-house tests help the diagnostic work if the serological tests do not give false positive reaction with *D. repens* antigen. For this reason it is a key question whether the commercially marketed *D. immitis* antigen kits may give false positive reactions in dogs infected with *D. repens* or not. The other question is whether the specificity of these tests may differ from the published data if the sera of dogs infected with both Dirofilaria species are screened.

Previous studies have reported that the current generation of heartworm antigen test kits is very highly specific (Courtney, 2001, McCall et al., Atkins, 2003). However, sometimes these kits may give false-positive result because of cross-reactions with other nematode antigens. It has been reported that serological cross-reactivity between *D. immitis* and *S. lupi* has been observed which might be due to shedding of circulating antigens with similar antigenicity detected by the assays (Aroch et al., 2015). In another study Schnyder and Deplazes (2012)

found cross-reactivity between *D. immitis* and *A. vasorum* antigens in dogs using in-clinic assays.

No detailed and reliable data are available about the cross-reactivity betwenn *D. immitis* and *D. repens.* In this study all of the evaluated test kits gave false positive reaction with sera of dogs infected with *D. repens*. Only one sample out of 15 sera became positive using three kits (WITNESS® DIROFILARIA, Fassisi® CanDiro and *FAST*est® HW Antigen) but the number of false positive reaction was five when DiroCHEK® was used. The specificity of WITNESS® DIROFILARIA was 93.3%. Similar result was published by Pakistanian scientists who had 96.87% specificity for this kit (Ranjbar-Bahadori et al., 2007). In their study 2 out of 16 dogs gave false positive results which were not infected with *D. immitis* according to necropsy. In another study the specificity of this antigen test ranged between 71% and 100% (McCall et al., 2001). Regarding DiroCHEK® kit its specificity was only 66.7% in this study which is lower comparing with data of McCall et al. (2001) and others (Gillis et al., 1984; Rhee et al., 1998) who found that the specificity of this kit ranged from 71% to 100%. Although the ELISA used in this study is a good test, it did not give 100% specificity in dogs infected with *D. immitis*. Thus, false positive and negative results may occur. Therefore it should be taken into account that an unknown number of dogs with travel anamnesis may have falsely been diagnosed positive due to *D. repens* cross-reactions, and are erroneously treated with melarsomine and/or macrocyclic lactones. From a practical point of views this finding is important in Hungary where currently more dogs are infected with *D. repens* than *D. immitis*. Based on these preliminary results there is serological cross-reactivity between *D. immitis* and *D. repens*. However, it cannot be excluded there is other reason for the false positive reactions because the dogs infected with *D. repens* had not been checked for *A. vasorum* and *S. lupi* infection, which parasite species are present in the country (Majoros et al., 2010; personal information given by Farkas). Therefore further studies are needed to answer this hypothesis. Another explanation for false positive result of *FAST*est®HW Antigen kit was stated by MEGACOR manufacturer. *Dirofilaria immitis* antigen may persist after natural or pharmacological death of adult worm. In these cases the results of antigen test remain positive for approximately 3-4 months. Therefore a second test is recommended 4 months later.

Taking together the results of 15 dogs infected with *D. immitis* and 15 dogs infected with *D. immits* and *D. repens* the specificity of three kits (WITNESS® DIROFILARIA Fassisi® CanDiro and *FAST*est® HW Antigen) is 96.7%, and 83.3% for DiroCHEK® . However, taking into account that three kits gave one, and DiroCHEK® resulted in five false positive reactions with *D. repens*, it is not known whether all the positive results of dogs infected with

both *Dirofilaria* species were true positivity or not. Based on the results none of the sera of uninfected dogs gave positive reaction with the evaluated kits which means no false positive reactions should be considered if *Dirofilaria* free dogs are screen. However, in these cases parasitosis caused by *A. vasorum* and *S. lupi* must be excluded.

It should be emphasized that the sensitivity was higher when serum samples from dogs infected with *D. immitis* were used alone, compared to the results obtained with samples from dogs with a double infection (73.3-86.7% vs 66.7-80.0%, respectively).

The reason for false negative result might be due to the small number of female worms when the antigen of *D. immitis* in infected dogs is below the detectable level (Atkins, 2003). Consequently, the success in the use of antigen tests is dependent on the amount of antigen released by mature adult female heartworms (Courtney, 2001). These kinds of tests detect antigens exclusively from female heartworms that are at least seven or eight months old but do not generally detect infections that are less than five months old (Lagrotteria et al., 2003). Therefore, it was exhibited that although the antigen tests are a valuable adjunct to diagnosis of *D. immitis* infection, the test were found to be less sensitive and prone to false negatives (Patton and McCracken, 1991).

The positive or negative predictive values of tests are dependent upon the prevalence of disease. For canine heartworm, false positive test results are more likely in dogs where the prevalence is low (Peregrine, 2005; Bowman, 2007). In an area that appears to be endemic for heartworm disease, a positive result with an antigen test would likely be a true positive.

Taking into consideration the results of this study without reliable data about the cross-reactivity between *D. immitis* and *D. repens* it can be stated that if the local veterinarians get positive result with any *D. immitis* antigen test they should exclude the infections caused by *A. vasorum* and/or *S. lupi*. Before starting the expensive treatment of heartworm infection in a dog they should confirm *D. immitis* infection with molecular biological study carried out in a special laboratory.

6. ABSTRACT

Dirofilaria immitis and *Dirofilaria repens* are important nematode species of dogs with overlapping endemic areas, especially in many parts of Europe including Hungary. For the detection of *D. immitis* infections, a variety of tests have been developed and marketed, however, they have not been evaluated for cross-reactions against circulating antigens of *D. repens* that has not been detected with marketed tests. The aim of this study was to compare the efficacy of four commercialized *D. immitis* antigen test kits using sera samples of naturally infected dogs with *D. immitis* or *D. repens* or both species.

Between 2014 and 2015, blood samples from domestic dogs kept in different counties of Hungary were subjected to this study. The infection of dogs was checked with two different PCR assays. There were 4 groups of sera tested: samples from 15 dogs with *D. immitis* infection (group A); samples from 15 dogs infected with *D. repens* (group B); samples from 15 dogs with mixed infection of *D. immitis* and *D. repens* (group C) and sera of 15 dogs without *Dirofilaria* infection (group D). The 60 sera samples were evaluated simultaneously using *FAST*est®HW Antigen (Diagnostik MegaCor, Hörzbranz, Austria), DiroCHEK® (Synbiotics Corporation, San Diego, USA), WITNESS®DIROFILARIA (Zoetis Inc., Lyon Cedex, France) and Fassisi®CanDiro (Fassisi, Göttingen, Germany).

Three *D. immitis* antigen kits gave false positive reaction with one of 15 sera of *D. repens* infected dogs (specificity was 93.3%). DiroCHEK® had worse specificity (66.6%), with this kit 5 out of 15 sera samples in this group of sera gave false positive results. If the results of dogs infected with only *D. immitis* and dogs infected with both *Dirofilaria* species are evaluated together, sensitivities of the WITNESS®DIROFILARIA, Fassisi® CanDiro and DiroCHEK® test kits are 76.7%, 83.3% and 83.3%, respectively. In this case *FAST*est® HW Antigen has the lowest sensitivity (70.0 %). It should be emphasized that the sensitivity was higher when samples from dogs infected with *D. immitis* were used alone, compared to the data obtained with serum samples of dogs with double infection.

Taking into consideration the results of this study without reliable data about the cross-reactivity between *D. immitis* and *D. repens* it can be stated that if the local veterinarians get positive result with any *D. immitis* antigen test first they should exclude the infections caused by *A. vasorum* and/or *S. lupi*. Before starting the expensive treatment of heartworm infection in a dog they should confirm *D. immitis* infection with molecular biological study carried out in a special laboratory.

7. REFERENCES

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7. ACKNOWLEDGEMENTS