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The evaluation of CHW Ag 2.0 test (Bionote) new to the Hungarian market in comparison to two previously reviewed in-clinic antigen tests

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Content

Introduction

Dirofilaria immitis is a vector-borne nematode species which is responsible for the heartworm disease in dogs which may be life threatening in severe cases. The parasitosis has a worldwide distribution, but it is predominantly present in tropical and subtropical areas [1]. The heartworm disease is transmitted to the hosts as third stage larvae which develop in the mosquito vectors. The disease predominantly affects the cardiopulmonary system and in more advanced cases the worms may enter the right ventricle of the heart. Although many dogs are infected with *D. immitis*, a large number of them do not show any clinic signs.

As the heartworm disease is endemic to many areas and can be of great clinical significance, veterinarians rely on rapid in-clinic tests for diagnosis. There is an array of methods that can and have been used for the diagnosis of canine heartworm infection. The important factors to consider when choosing a testing method are importantly the accuracy, functionality, cost, and suitability to the practice. When speaking of accuracy, the sensitivity and specificity of a test are important [2].

There are various methods that can be used to diagnose heartworm infections. The most notable of these methods are the detection of microfilariae (mfs), serodiagnosis and the detection of DNA. There are a number of additional methods such as blood chemistry and imaging that can assist in determining the severity of the disease.

The diagnosis of heartworm infection by the detection of mfs can be done with techniques such as the modified Knott test, the filtration method or histochemical staining [3]. It should always be taken into consideration that occult infections may occur, where there is an infection with adult *D. immitis* and there are no circulating mfs. This may occur if the test has been conducted during the prepatent period, if there is an all-male heartworm infection or due to drug induced sterility of the adult heartworms. The examination of mfs in blood smears is a possible method of morphological differentiation between *D. immitis* and *Dirofilaria repens* in the countries where both species occur [4].

Serological testing is used for the detection of circulating antigens of adult female worms. These tests such as enzyme linked immunosorbent assay (ELISA) and immunochromatographic tests (ICT) are now commonly used in veterinary practice for the detection of heartworm infection. The antigen tests have mostly replaced the traditional microfilaria detection methods as they

are more sensitive and specific. Another advantage of the antigen tests is that they are still able to detect heartworm infection during or after the use of macrolide drugs which have an antimicrofilarial effect [2]. Unfortunately, the sensitivity of the antigen tests may be decreased in the case of a low worm burden or a low number of female worms [5]. Although the antigen tests are specific to *D. immitis*, cross-reactions can occur with antigens of other nematodes such as *D. repens*, *Angiostrongylus vasorum* and *Spirocerca lupi* [6]. There are currently no available commercial tests for the detection of antibodies against *D. immitis*, although Joekel et al. [7] presented a monoclonal antibody-based sandwich-ELISA for the detection of antibodies against *D. immitis* and *D. repens* in the sera of dogs.

The aim of this study was to compare the results of the Antigen Rapid CHW Ag Test Kit 2.0, which has recently become available in Hungary to the WITNESS® Heartworm test and the DiroCHEK Canine Heartworm Antigen Test Kit by testing blood samples of dogs infected with *D. immitis* or *D. repens.*

Literature review

1. Biological attributes

1.1. Taxonomy and morphology

The nematodes of genus *Dirofilaria* belong to family *Onchocercidae* [8]. The genus *Dirofilaria* is divided in two subgenera, *Dirofilaria* includes *Dirofilaria* i*mmitis* and the subgenus *Nochtiella* includes *Dirofilaria repens* [9].

The first stage larvae (L1) of *D. immitis*, rather known as the mfs are 270-365μm long and 6.7-7.1μm wide [10]. Whereas the adults are cylindrical, slender greyish white worms with a transversely striated cuticle. The males measure about 12-16cm in length and the females about 25-30cm in length. The posterior end of the males is coiled whilst the posterior of the females is straight, large and rounded [11]. They have filariform oesophagi, anal and excretory pores. The male has a seminal vesicle and testis whilst the female has an ovary and oviduct. The adult heartworms are usually located in the pulmonary artery and the right ventricle of the heart. The heartworms are able to migrate to sites other than the pulmonary vasculature in the canine host. Some of the described atypical sites are the muscles; brain; spinal cord and the anterior chamber of the eye. The parasites are also able to migrate into the aortic bifurcation and have even been observed as distally as the digital arteries [12].

1.2. Life cycle

The mfs are ingested by female mosquitoes when they take up blood from infected animals in which they develop into infective third instar larvae (L3) within 10 -14 days [13]. The L3 enter the host via the small puncture wound created by the mosquito's stylet. At around the 3rd day, the L3 moult from into L4 [14] and migrate towards the abdomen and thorax where they undergo their final moult between days 50-70 after infection [3]. The juvenile worms begin to reach the heart within a few weeks. Once the immature worms have entered the vascular system they will mature into adult worms and reach sexual maturity at about 120 days post infection. In this period the worms grow drastically in length from 2-4cm to 15-30cm, with the females being the larger of the species. After mating, mfs are released into the host's bloodstream by the female worms [15]. The first circulating mfs can be found in blood of infected dogs as early as 6 months post-infection although in most dogs they are found first

between 7-9 months post-infection [14]. In cats the prepatent period is longer than in dogs. The first circulating mfs can be seen at 7-8 months in cats versus 6 months in dogs [16]. The worms will generally live for 3 to 4 years in cats and in many cases may clear without treatment [16].

1.3. Pathophysiology and clinical signs

There are four classes of heartworm disease, with the first being asymptomatic. The second class includes mild to moderate signs such as coughing, exercise intolerance and abnormal lung sounds. The third class includes the severe signs such as dyspnoea, abnormal heart and lung sounds, hepatomegaly, syncope ascites and possibly death. The fourth class is called Caval syndrome which includes hemoglobinemia and haemoglobinuria [17]. Cavel syndrome occurs when the adult worms migrate in a retrograde manner from the pulmonary arteries to the right side of the heart and back into the venae cavae [12].

The severity of a heartworm infection and the clinical signs present may depend on a number of factors, these were described by Atkins [18] as the following; the number of worms present; the immune response of the infected animal; the duration of infection; and the activity level of the animal. The presence of heartworms in the vessels within the heart itself causes irritation and inflammation which may be further exacerbated by the hosts immune response. *D. immitis* typically live for 3–5 years in a host's body, the irritation caused by these long-term infections will eventually lead to scarring of the blood vessels [18].

1.4. Host species

In a study conducted by Trotti et al. [9] on the canine hosts in the Americas, the parasite was found in a variety of species such as the domestic dog; grey wolf; coyote; red fox; grey fox and the maned wolf. In other parts of the world infection has been reported in the jackal, the raccoon dog, the dhole, and the African wild dog [9]. The same study mentioned both domestic and non-domestic feline hosts, as well as several species that have nonpatent infections. These species have been identified as primates, deer, beavers, muskrats, horses, wolverines, red panda, raccoons, bears, seals, sea lions and domestic ferrets.

1.5. Prevalence of *D. immitis* in Europe

According to Genchi et al. [19] *D. immitis* which was previously only endemic in southern European countries, is now an emerging disease and is spreading towards eastern, central and western Europe. There are multiple factors that could possibly contribute to this spread including; climate change; changing human activity; movement of dogs or the introduction of new mosquito species which are able to act as vectors [20]. Unlike human malarias which can only be transmitted by *Anopheles* spp., *D. immitis* has been shown to develop in more than 60 mosquito species of different genera around the World [16].

As the geographical distribution of heartworm continues to expand, wildlife populations are acting as reservoirs. A study was conducted by Penezić et al. [21] on the prevalence of *D. immitis* in wild carnivores in Serbia. It was found that between the years of 2009 to 2013 the prevalence was the highest in golden jackals (7.32%) and in wild cats (7.69%). The movement and the presence of stray dogs is another highly important factor in the spread of heartworm due to the exposure to both wildlife and pet animals and the lack of prophylaxis.

Stoyanova et al. [22] conducted a study on 80 stray dogs from different parts of the city of Sofia, in Bulgaria. The study concluded that the prevalence of *D. immitis* in the canine population in the studied area was 31.25 %. In a study conducted by Mircean et al. [23] on various vector-borne infections in Romanian dogs the prevalence of *D. immitis* in the population was much less than (3.3%) that of the Bulgarian study. The vast difference in the prevalence of *D. immitis* between these bordering countries could be due to a number of factors such as stray dog populations, differences in chemoprophylactic drug administration or variable sample groups used in the studies.

Farkas et al. [24] conducted the first comprehensive study on vector-borne pathogens in dogs within Hungary. For this study 1305 blood samples were collected and screened for the detection of circulating antibodies of *Ehrlichia. canis, Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *D. immitis* antigen using SNAP® 4Dx (IDEXX Laboratories). The results showed that the prevalence of *D. immitis* was 2.4% in the population. In a second Hungarian study, 344 blood samples were collected from randomly selected dogs from 19 counties of Hungary. The dogs lived exclusively outdoors, had never travelled and had not been previously examined or treated for *Dirofilaria* infections [25]. This examination for *Dirofilaria*

was conducted with a modified Knott's test, DiroCHEK antigen test, as well as by multiplex and conventional PCR. The results showed that 8.1% of the dogs were infected with *D. immitis*, 11.1% were infected with *D. repens* and 3.2% had coinfections.

2. Diagnosis of the canine heartworm disease

For the purpose of accurate diagnosis, it is important to take many factors into account such as the timing of infection, the use of prophylaxis or adulticide treatment and potential co-infections in the area. Much emphasis is put on both the sensitivity and the specificity of each test. When talking about sensitivity (Se) and specificity (Sp), it is important to understand what is meant by each term. The Se of a test will measure how often a test correctly generates a positive result when the animal is truly positive for the condition that is being tested. A test with 100 percent Se will accurately show all of the positive animals in the tested population without any false negative results. The Sp of a test measures the ability of a test to correctly show a negative result in all of the patients that do not have the disease or condition that is being specifically tested for. If a test kit were to have 100 percent Sp, it would not show any false positive results for the condition [26].

2.1. Detection of microfilariae

There are various methods that can be used to diagnose *D. immitis*, the first of which involve the detection of mfs. The mfs can be detected in blood samples by microscopy using a direct blood smear; the Knott test; modified Knott's test or membrane filtration to concentrate the mfs [27]. According to the American Heartworm Society [28] the modified Knott's test is the recommended test for circulating mfs. This method involves diluting 1 ml blood in 9–10 ml formalin (2%), to lyse the red blood cells to improve readability, allowing for morphology-based identification. The formalin also helps to preserve the mf for later examinations [29]. The filter technique utilizes a mixture of lysine solutions and EDTA-blood, which is then mixed with methylene blue solution and forced through a filter pad. The pad is then examined microscopically for mfs [30].

Histochemical staining of mfs is useful in the identification and differentiation of the mfs of the two *Dirofilaria* species. Once smears are made and fixed, they are stained with the acid phosphatase histochemical stain which precipitates in different bands or spots depending on the

species. *D. immitis* shows two distinct bands which are associated with the anal and excretory pores and *D. repens* only shows one coloured band, which is associated with the anal pore [31].

It is possible for dogs to have mfs of *D. immitis* but remain antigen-negative regardless of the antigen test that is used. This may be due to a number of factors such as the misidentification of the circulating mfs as the mfs of *D. repens* or other parasites, the possible transplacental transmissions of mfs to pups from the dam, iatrogenic inoculation in a blood transfusion, or the death of the adult worms due to adulticide treatment but persistence of mfs [32]. Dogs that are receiving macrolides for the prevention of heartworm will become amicrofilaremic from about 3-7 months after therapy, making microfilaria detection methods unreliable [2]. Current macrolide preventatives such as ivermectin, milbemycin oxime, moxidectin and selamectin result in a significant clearance of mfs from the blood, this can be a cause of false negative results [3].

2.2. Serodiagnosis

There has been considerable difficultly with the detection of *D. immitis* antibodies in the diagnosis of canine heartworm disease. One of the first serological tests used in the detection of heartworm infection was the indirect fluorescent antibody (IFA) assay, which detected antibodies for microfilarial surface antigens. The test was only able to detect antibodies in dogs that showed a hypersensitivity reaction to the mfs. This led to high numbers of false-negative results [2]. Another significant problem with the use of antibody testing in dogs is the poor Sp of tests due to a high degree of homology with epitopes of particular helminth species. This leads to many false positive results and cross-reactions in dogs infected with helminth species such as *Toxocara canis, Angiostrongylus vasorum Acanthocheilonema* spp. and *Dipetalonema* spp. [7]. Due to the difficulty in the detection of antibodies the detection of antigens is the preferred serodiagnosis.

The antigens, a series of related proteoglycans, are produced by the uterus of the adult female worm. Antigenemia can be detected at about 5 months post-infection, earlier than the appearance of mfs in the blood [2]. The currently available patient-side antigen tests are Enzyme-linked immunosorbent assay (ELISA), Immunochromatography and Hemagglutination tests. As these tests rely on the presence of sexually mature female worms,

they can produce false negative results if the infection has been present for less than 5-6 months, there is a low worm burden or there are only male worms present [32].

A study conducted by Schnyder and Deplazes [6] was able to confirm that *Angiostrongylus vasorum* may cause a cross reaction in commercially available antigen test kits for *D. immitis*. Their results showed false positive results in the *D. immitis* test kits with the sera of dogs infected with *A. vasorum*. The adult stages of both *D. immitis* and *A. vasorum* are located in the heart and the pulmonary arteries of their host's and these nematodes share overlapping endemic areas in southern Europe. For these reasons it is important that simultaneous diagnostic tools are used to avoid misdiagnosis [6]

The cross-reactivity between *D. immitis* and *D. repens* has been discussed in previous studies, stating that there have been false positive results on heartworm antigen tests in cases of dogs naturally infected with *D. repens* [33]. During an in vitro study conducted by Venco et al. [34], it was observed that *D. repens* may release more detectable antigens than the other worms used in the study. This suggests that *D. repens* infection can lead to false-positive results on heartworm antigen tests, in a similar manner to that of *A. vasorum*.

The formation of antigen-antibody complexes is another cause of false results. It has been proposed that heat treating the sera samples prior to testing may help to release bound antigens and assist in the detection of infection particularly in dogs that have received heartworm prophylaxis [35]. Due to the various potential causes of false positive and false negative reactions, dogs with clinical signs indicative of canine heartworm disease but with negative results on an antigen test should be tested further. Methods which can be used to confirm the results of an antigen tests include the detection of mfs, DNA detection methods and imaging such as x-ray or echocardiography.

The first patient-side test for heartworm utilised ELISA technology. ELISA assays are either microtiter or membrane based. A protein specific for the antigen that is being tested for is bound to the membrane or microwell. Once the sample solution is added, binding of the antigen will occur. Once washed a solution is added containing antigen-specific antibody conjugated to an enzyme. This antibody will bind to the antigen if it is present and cause a change in colour [2].

In comparison to the ELISA technology, the more recently developed antigen tests utilise immunochromatographic (ICT) assays [36]. A study conducted by Martini et al. [37] compared the results of five haematological tests (filtration, direct smear, modified Knott, clotted blood and capillary tube) and three commercial ELISA kits (PetChek, Diasystems, Uni-Tec). This study showed that whilst all of the tests showed a correlation between the worm burden and the sensitivity of the test, the ELISA methods were better at detecting the cases with low worm burdens. A benefit of the microtiter plate ELISA tests is that they are able to provide graded test reactions with the strength of the colour reaction correlation with the degree of worm burden. The microtiter plate ELISA is also considered to be the most sensitive form of antigen testing, detecting up to 85.7% of dogs with a single female adult worm and 100% of infected dogs with at least three adult female worms [32]. Although the commercial ELISA test kits may be one of the more sensitive testing methods in positively identifying low worm burdens, the method is not particularly quick when used patient-side.

Immunochromatography is a combination of an immunoassay and chromatography [38]. It is used for a variety of applications within the medical, veterinary and industrial industries. The assays can be used either for the detection of antibodies or antigens. Depending on the assay there will be either antigens or antibodies immobilized on the nitrocellulose membrane in a line or cartridge. With the addition of a positive sample and the capture of either the antigen or antibody there is then the addition of an enzyme-labelled conjugate and substrate. This leads to a colour reaction appearing on the line. As lateral flow immunoassays created for clinical use are usually double sandwich assays, for the detection of antibodies the test line will contain immobilized antibodies [38].

The WITNESS Heartworm test (WIT, Zoetis LLC) has been re-licenced twice, with the most recent re-licensing in 2015. A study was done using canine plasma to compare the WITNESS test to another patient side heartworm test; the single nucleotide polymorphism (SNP) test. In this study the results for the WITNESS test showed 95% sensitivity and 96.4% specificity [39]. The test utilizes antigen-specific antibody labelled with colloidal gold. The visualisation of the heartworm antigen is based on the on the use of coloured colloidal gold particles instead of the enzyme-linked colour change reaction or a haemagglutination. A blood sample is added to the test kit with a dropper, and the blood will flow along the strip to the “patient line” or test line where antibodies specific to *D. immitis* are immobilised. If there are heartworm antigens present in the sample, they will be captured by the antibodies present on the “patient line”. A second

antibody is tagged with colloidal gold particles. This tagged antibody is able to bind to the captured heartworm antigen causing a visible line to develop. The test has a control line that contains another immobilized antibody, this line will always show a positive reaction if the test has been run correctly [40].

Lee et al. [41] conducted a study on an in-clinic, instrument-based rotor utilizing the colloidal gold agglutination method. The VetScan VS2 is used for the analysis of chemistry and electrolyte profiles, as well as tests such as; T4; Bile Acids; TCO2; and Canine Heartworm. It is useful in clinical practice as a heartworm test can be conducted along with renal and hepatic evaluations without any extra laboratory work. The VetScan VS2 does not appear to be as accurate in detecting heartworm infections with low worm burdens [41]. Therefore, in patients where there is a suspicion of Heartworm infection it may be better to use a testing method that is specific only to heartworm disease.

2.3. Detection of DNA

The detection of the heartworm disease by the detection of DNA can be done by using Polymerase Chain Reaction (PCR) technology or Western blot analysis. Both single species and multiplex PCR assays have been used for the identification of specific microfilaria in peripheral blood samples. Amplification of DNA from circulating microfilaria in a PCR assay gives reliable positive results show high sensitivity. A disadvantage of the PCR testing is that it is only able to detect patent infections when circulating microfilaria are present [32]. PCR testing is able to accurately distinguish between infections from *D. immitis* and *D. repens* [27].

There are species specific PCR assays for the identification of single species, multiplex PCR assays and PCR- restriction fragment length polymorphism (RFLP) assays for simultaneous detection of the different *Dirofilaria* species in both the mosquito vectors and the host's blood. These methods are based on either two separate reactions or the use of post-PCR steps such as RFLP which increases the time of the procedure. A single single-step multiplex PCR assay which is able to discriminate between the dirofilarial species is a useful tool in areas which are co-endemic to both species. It was shown that in samples with as little as four microfilariae per ml, the multiplex PCR assay was able to detect and differentiate between *D. immitis* and *D. repens* [42].

Various studies have been conducted on the possible use of Western blot (WB) analysis in the diagnosis of heartworm disease. A study was conducted by Oge et al. [43] in order to show the presence of serologically identifiable antigenic components in the protein extract of adult *D. immitis*. It was found that there are specific antigens of molecular weights of 14 and 16 kDa on the mf surface. These molecules show a reaction with sera from dogs infected with *D. immitis* but not with sera from uninfected dogs or sera from dogs that are infected with potentially cross-reactive nematodes [43].

Materials and methods

The tested samples

Altogether 61 blood samples were used in this study. The samples were collected from dogs and examined using PCR technology for *Dirofilaria* spp. Twenty-three samples were positive for *D. immitis* and 18 samples were positive for *D. repens*. A further 20 sera samples were also tested, which were negative for both *D. immitis* and *D. repens*. The marked serum samples were stored in a deep freezer before the serological studies were carried out in the Department of Parasitology and Zoology.

Serological methods

The **CHW Ag Test Kit 2.0** (**Figure 1**), developed by Bionote is a relatively new test kit in comparison to other rapid test kits for *D. immitis*. It is a chromatographic immune assay, that utilizes the highly selective antibodies to canine *D. immitis* antigens. These are used to capture the antigens and to act as an indicator in the assay. Bionote states that the CHW Ag 2.0 test kit has a sensitivity of 99.5% and a specificity of 94.0% [44].

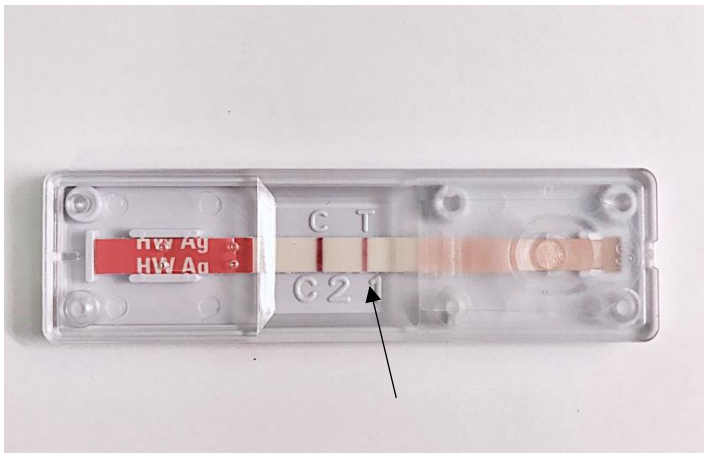

FIGURE 1. The arrow indicates the positive *D. immitis* result on the CHW Ag Test Kit 2.0

WITNESS Canine Heartworm Antigen Test Kit (**Figure 2**), developed by Zoetis is based on rapid immune migration technology. The test uses sensitized colloidal gold particles which bind to the antigen present in the sample which is then able to migrate along the strip. This complex is then caught in the sensitised reaction zone where it forms a clear pink or purple band. There is a clearly visible control band to ensure that the test was performed correctly [40]. Zoetis states that the sensitivity of the test is 96.6% (CI:91.0%-99.0%) and it's specificity is 99.0% (CI:96.0%-100.0%).

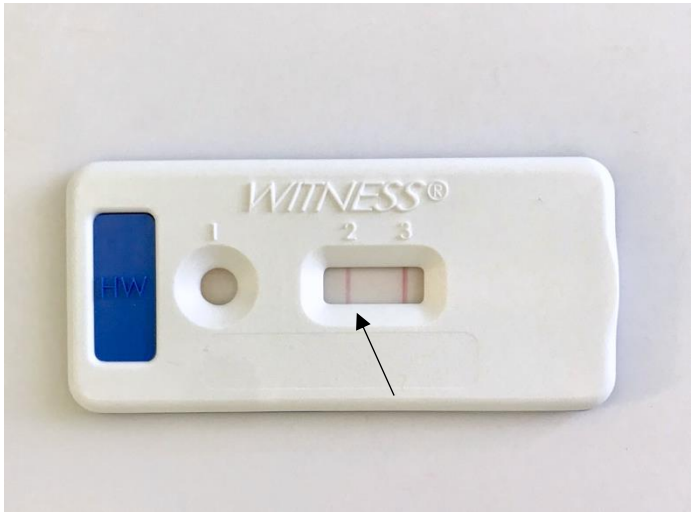

FIGURE 2. The arrow indicates the positive *D. immitis* result on the Witness *Dirofilaria* test

DiroCHEK Canine Heartworm Antigen Test Kit (**Figure 3**) developed by Zoetis is a direct Sandwich ELISA. The wells are coated with antibodies against *D. immitis*, a second antibody is labelled with the horseradish peroxidase enzyme. The antigens are bound by the antibody coated well and the enzyme-linked antibodies to form a complex. In the presence of *D. immitis*, a blue colour develops [45]. Zoetis states that both the Se and Sp of the DiroCHEK Canine Heartworm Antigen Test Kit are 100% when there are more than 3 female heartworms present.

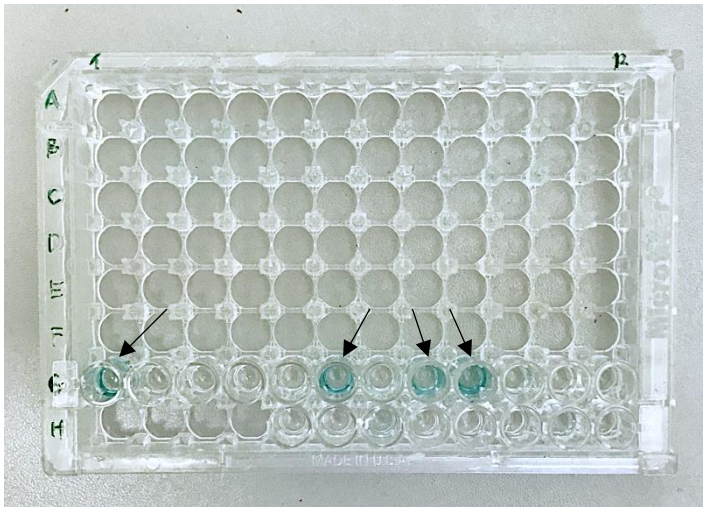

FIGURE 3. The arrows indicate the blue colour reaction is seen in the samples which are positive for *D. immitis* using the DiroCHEK Canine Heartworm Antigen Test Kit

All three test kits were used prior to their respective expiration dates and they were kept and used according to the instructions provided in their respective package inserts.

Statistical evaluation of the results

The sensitivity of each test in this study was calculated as 100% x TP/(TP+FN). The specificity was calculated as 100% x TN/(FP+TN). TP is True positive, TN is True negative, FP is false positive and FN is false negative. The accuracy is calculated as (TP+TN)/(TP+TN+FP+FN) [46].

Results

Out of 23 serum samples of *D. immitis* infected dogs confirmed with PCR, 20 samples gave positive reactions and 3 samples were negative using the CHW Ag 2.0 test (**Table 1**). The results were the same for the WITNESS test. There were 20 positive and 3 negative samples, respectively. The 3 samples with negative results were the same in both the CHW Ag 2.0 and WITNESS tests. Using the third test, the DiroCHEK all 23 samples as positive (**Table 1**).

Regarding the 20 serum samples that were confirmed to be negative for both *D. immitis* and *D. repens* by PCR, all 20 were negative with the three test kits (**Table 1**).

During the serological examination of 18 serum samples collected from 18 dogs infected with only D. repens confirmed by PCR, 13 out of 18 gave negative reactions and 5 gave positive reactions the the CHW Ag 2.0 test (**Table 1**). In both the WITNESS and the DiroCHEK tests 15 samples were found to be negative and 3 were positive (**Table 1**).

TABLE 1. The results of the antigen tests for the serum samples confirmed by PCR to be either *Dirofilaria* negative, *D. immitis* positive or *D. repens* positive.

	Dirofilaria immitis negative (20)		*Dirofilaria immitis* positive (23)		*Dirofilaria repens* positive (18)	
	Positive	Negative	Positive	Negative	Positive	Negative
WITNESS	0	20	20	3	3	15
CHW Ag 2.0	0	20	20	3	5	13
DiroCHEK	0	20	23	0	3	15

TABLE 2. The results of the antigen tests including the sensitivity, specificity and accuracy all calculated at a 95% confidence interval.

	Number of samples						
Test kit	True +	True -	False +	False -	Sensitivity (%)	Specificity (%)	Accuracy (%)
WITNESS	20	35	3	3	86,96 (66.41-97.22)	92,11 (78.62-98.34)	90,16 (79.81-96.30)
CHW Ag 2.0	20	33	5	3	86,96 (66.41-97.22)	86,84 (71.91-95.59)	86,89 (75.78-94.16)
DiroCHEK	23	35	3	0	100 (85.18-100)	92,11 (78.62-98.34)	95,08 (86.29-98.97)

As seen in **Table 2** and **Figure 4** the CHW Ag 2.0 and the WITNESS test had the same level of sensitivity (86.96%) at a 95% confidence interval. The sensitivity of the DiroCHEK test was 100%. The specificity of the CHW Ag 2.0 test was lower (86.84%) compared to the other two antigen tests, where the obtained specificity was 92.11% (**Table 2**). The accuracy of the CHW Ag 2.0 the lowest (86.89%) of the three tests (**Table 2**, **Figure 4**). The obtained accuracies of the WITNESS and DiroCHEK were 90.16% and 95.08% respectively (**Table 2**, **Figure 4**).

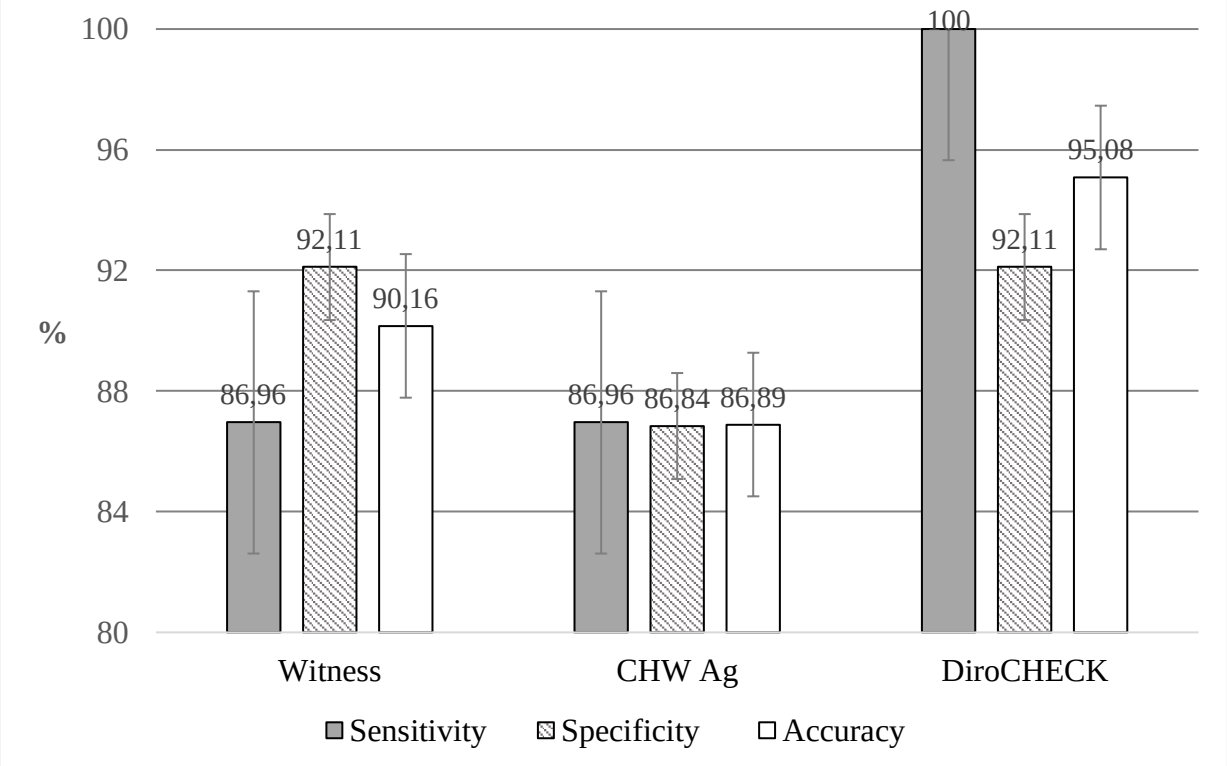

FIGURE 4. Bar graph indicating the sensitivity, specificity and accuracy of the *D. immitis* test kits

Discussion

The goal of this study was to evaluate the performance of the CHW Ag 2.0 test in comparison to the WITNESS and the DiroCHEK antigen tests. When comparing the results from the CHW Ag 2.0 test to both the WITNESS and DiroCHEK tests, results showed that the sensitivity of the CHW Ag 2.0 test was comparable to that of the WITNESS test. It was observed that the DiroCHEK had the highest sensitivity. It has been noted in previous studies that ELISA assays such as the DiroCHEK have high sensitivity in terms of the detection of *D. immitis* infection in the case of low worm burdens [32].

As the three results that gave negative reactions in both the CHW Ag 2.0 and WITNESS tests were confirmed to be positive for *D. immitis* with PCR, these are taken as false negatives. Potential causes of false negative results are a low worm burden, testing too early for antigens to be apparent, an infection with only male worms or due to the previous use of prophylactic treatment [47]. Another proposed reason for false negative results in previous studies was that in dogs possessing high amounts of antibody against *D. immitis,* the antibodies can mask the antigen detection [48]. It has been proposed that the heat treatment of samples prior to testing can disrupt the antigen-antibody complex which may assist in detection of the antigens [32]. This heat treatment is not recommended in areas where *D. repens* and *A. vasorum* are also present as heat treating the samples causes an increase in cross-reactivity and therefore decreases the specificity of the tests [34].

In the case of this study, it is possible that the three false negative reactions are due to low worm burdens in the sera as the DiroCHEK ELISA assay gave weak positive reactions seen by pale blue colour reactions for the three samples.

The CHW Ag 2.0 test had the lowest specificity out of the three antigen tests, this was due to a higher number of false positive test reactions than in the other two tests. A reaction was taken as a false positive if the *D. immitis* antigen test gave a positive rection to a serum sample infected with *D. repens* confirmed with PCR. Of the 18 serum samples infected with *D. repens,* both the WITNESS and DiroCHEK tests gave 3 positive reactions and the CHW Ag 2.0 test gave 5 positive reactions. False positive results on *D. immitis* antigen test kits can occur due to cross reactions with *D. repens* and *A. vasorum* [34]. This study emphasised the cross-reaction with *D. repens* which is endemic to Hungary [25].

Accuracy is the proportion of true positive results, including both the true positive and true negatives. In terms of and accuracy the CHW Ag test achieved lower results than the other two antigen tests. All three of the antigen tests had accuracies >80%. The positive predictive values indicating the probability that a dog infected with *D. immitis* will have a positive test result, were 80.00% for CHW Ag 2.0, 86.96% WITNESS and 88.46% for DiroCHEK. The negative predictive values give the probability that a dog which is free from heartworm disease will have a negative test result. The negative predictive values were 91.67% for the CHW Ag 2.0, 92.11% for WITNESS and 100% for DiroCHEK. The predictive values in this study are likely inflated due to the high prevalence of infection of *D. immitis* within the sample group that does not accurately represent the canine population of the tested area.

The test performance of all three tests were slightly inconsistent with the reported sensitivity and specificity of each assay. The CHW Ag 2.0 has a reported sensitivity of 99.5% and specificity 94.0% [44] whereas the sensitivity (86.96%) and specificity (86.84%) established in this study were lower. WITNESS has reported sensitivity of 96.6% and specificity of 99.0%. Both the sensitivity (86.96%) and specificity (92.11%) of the WITNESS test were slightly lower than the reported values [45]. The DiroCHEK has a reported sensitivity of 100% which the results of this study agreed with. the reported specificity of DiroCHEK is 100% [45] whereas the results of this study produced a slightly lower specificity (92.11%).

The CHW Ag 2.0 and the WITNESS tests are marketed as rapid screening tests. In the case of a screening test the sensitivity is of greater significance than the specificity [49]. The American Heartworm Society recommends additional confirmation of positive rapid screening with a plate antigen ELISA such as the DiroCHEK and testing for mfs using the Knotts test [28]. Additional diagnostic methods such as radiography and echocardiography are also recommended for ascertaining clinical status. This is of much importance when the history and the health of the dog do not coincide with the test results. Heartworm treatment should not begin until the diagnosis has been confirmed [47].

A limitation in this study was that the serum samples were stored in the freezer prior to testing for varying lengths of time. Due to the difficulty in obtaining a high number of samples from infected dogs, the study was conducted with stored frozen samples. According to the Bionote, CHW Ag 2.0 manufacturer's product insert, serum samples should not be frozen before use as severely haemolysed samples may alter test results [44].

A second limitation of this study was the size of the tested population. It is suggested that in order to narrow the confidence limits of the sensitivity and specificity values generated in a test, a minimum of 100 dogs should be used in each test group [50]. The size of each test group varied in the study as only sera from dogs that were naturally infected was used.

Conclusion

In this study it was found that all three commercial antigen tests were highly accurate, sensitive and specific. The CHW Ag 2.0 test kit which is new on the Hungarian market, matched the WITNESS test in terms of sensitivity but had a lower performance than the WITNESS and the DiroCHEK test kits overall. All three test kits showed a cross-reaction with *D. repens*. This implies that in areas where both *D. immitis* and *D. repens* are endemic, a positive antigen test result should be supported by further evidence of heartworm disease.

Abstract

The CHW Ag 2.0 test (Bionote) has recently become available for commercial use in Hungary. The test kit was compared to two other commercially available antigen test kits, the WITNESS® Heartworm Canine Heartworm Antigen Test Kit (Zoetis) and DiroCHEK (Zoetis). Blood samples were collected from 61 dogs and examined using PCR for *Dirofilaria* spp. Twenty-three samples were positive for *D. immitis* and 18 samples were positive for *D. repens*. A further 20 sera samples were also tested, which were negative for both *D. immitis* and *D. repens*. The samples were examined with the three antigen test kits in accordance to their respective package inserts.

The statistical evaluation of each test included the sensitivity, specificity, accuracy, positive and negative predictive values. Both the CHW Ag 2.0 and WITNESS tests yielded 3 false negative results and their sensitivity ranged from 66.61-97.22% at a 95% confidence interval. The DiroCHEK had a higher sensitivity range of 85.18-100% at the 95% confidence interval. During the serological examination of the 18 serum samples which were infected only with *D. repens*, the CHW Ag 2.0 gave 5 false positive reactions. Both the WITNESS and the DiroCHEK gave 3 false positive reactions. Regarding the 20 serum samples that were confirmed to be negative for both *D. immitis* and *D. repens* by PCR, all 20 of the samples were negative with the three test kits. The specificity of the CHW Ag 2.0 ranged from 71.91-95.59% at the 95% confidence interval. Whereas both the WITNESS and DiroCHEK had a specificity ranging from 78.62-98.34%.

The test performance of all three tests were slightly inconsistent with the reported sensitivity and specificity of each assay. Overall, the CHW Ag 2.0 matched the WITNESS test in terms of sensitivity but was outperformed by the other two antigen tests kits in terms of specificity and accuracy. All three test kits showed a cross-reaction with *D. repens*. Therefore, in areas that are endemic to both *D. immitis* and *D. repens*, additional diagnostic methods are recommended. This is of much importance when a positive result is seen on a rapid antigen test but the history and health of the dog do not coincide with the test results.

Összefoglaló

A CHW Ag 2.0 teszt (Bionote) a közelmúltban Magyarországon is kereskedelmi forgalomba került. A tesztet két másik, kereskedelemben kapható antigénteszt-készlettel, WITNESS® Heartworm Canine Heartworm Antigen Test Kit-tel (Zoetis) és a DiroCHEK-kel (Zoetis), hasonlították össze. Vérmintákat vettünk 61 kutyától, és PCR-rel vizsgáltuk *Dirofilaria*-fajok okozta fertőzöttségre. Huszonhárom minta volt pozitív *D. immitis*-re és 18 minta *D. repens*-re. További 20 szérummintát is teszteltünk, amelyek mind a D. immitis, mind a *D. repens* tekintetében negatívak voltak. A mintákat a három antigénteszt-készlettel a hozzájuk tartozó betegtájékoztatónak megfelelően vizsgáltuk.

Az egyes tesztek statisztikai értékelését az érzékenység és a specificitás, a pontosságot a pozitív és negatív prediktív értékek alapján végeztük. A CHW Ag 2.0 és a WITNESS teszt 3 fals negatív eredményt adott, érzékenységük 66.61-97.22% volt. A DiroCHEK érzékenységi tartománya magasabb, 85.18-100% volt. A 18, csak *D. repens*-szel fertőzött állat szérummintájának szerológiai vizsgálata során a CHW Ag 2.0 öt fals pozitív reakciót adott. A WITNESS és a DiroCHEK 3-3 fals pozitív reakciót adott. Ami a 20, nem fertőzött kutyából vett szérummintát illeti, ezek mindegyike negatív eredményt adott a három tesztkészlettel. A CHW Ag 2.0 specificitása 71.91-95.59%, a WITNESS és a DiroCHEK esetében ez az érték 78.62-98.34% volt.

Mindhárom teszt kapott eredményei kismértékben eltértek a közölt adatoktól. Összességében a CHW Ag 2.0 megfelelt a WITNESS tesztnek az érzékenység tekintetében, de a specifitás és a pontosság tekintetében felülmúlta a másik két antigéntesztet. Mindhárom teszt keresztreakciót mutatott a *D. repens*-szel. Ezért azokon a területeken, ahol a *D. immitis* és a *D. repens* endemikusan fordul elő, további diagnosztikai módszerek használata javasolt. Ennek akkor van nagy jelentősége, ha az antigén gyorsteszt pozitív eredményt ad, de a kutya története és egészségi állapota nincs összhangban a teszteredményekkel.

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