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Buffy coat smear or Knott's test: which to choose for canine microfilaria screening in field studies?

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Background: In recent years, an increasing number of cases of canine dirofilariasis have been reported worldwide. However, the rate of infection in dogs is largely unknown in many remote areas, and the importance of field studies for determination of the prevalence of canine dirofilariasis in such areas is well recognized. The detection of microfilariae by the modified Knott's test (MKT) is a recommended screening method for canine dirofilariasis.
Objectives: The purposes of this study were to compare the diagnostic sensitivity of the MKT with the buffy coat smear method (BCS), and to evaluate the utility of these 2 methods under field study conditions.
Methods: One hundred and fifty dogs of the Maio Island of Republic of Cabo Verde, were screened for microfilariae using MKT and BCS. The results of the 2 methods were generated in a blinded manner and
statistically compared.
Results: The detection rate was 4.67% with the MKT and 5.33% with BCS, which is statistically not different. The latter allowed a morphologic identification of Dirofilaria repens (later confirmed by molecular biology methods) and an estimation of parasite load, which varied from 15 to 185 microfilariae/mL.
Conclusions: The methods MKT and BCS were comparable in terms of diagnostic sensitivity. However, the BCS was technically less demanding and produced permanent preparations, in which co-infection with other hematologic pathogens can easily be assessed. Overall, this method is well suited to assess microfilariae in a large number of animals, and it could replace the MKT in studies devoted to dirofilariasis.

Introduction

Almost 4 centuries have passed since the first description of canine filariae, but dirofilariasis remains a subject of intense study in veterinary medicine.[1] Nowadays, 2 forms of dirofilariasis are considered: the cardiopulmonary form or so-called heartworm disease caused by *Dirofilaria immitis,* and the subcutaneous form caused by *Dirofilaria (Nochtiella) repens*.[2] Both forms are transmitted by mosquitoes of the *Culicidae* family. Although being fully adapted to canine hosts, reports of feline and human infections have increased in number in recent years.[1,2]
Global warming has caused an expansion of dirofilariasis[2], and a growing number of autochthonous cases have been diagnosed in the northern part of Europe and throughout the world.[1] Coupled with

the importation of animals and the increase in traveling pets during vacations, there is an enhanced risk for new autochthonous cases of canine dirofilariasis in regions that were previously considered to be free of the disease.[1] For instance, an incidence of 7.7% microfilaria infested dogs imported from the Mediterranean region to Germany has been reported.[3] Moreover, there are still large areas in the world, especially in Africa, where the prevalence of canine dirofilaria infection is largely unknown.[1] Therefore, screening for dirofilaria infection in dog populations throughout the world is relevant, not only to avoid international dissemination by the importation of pathogens but also to implement national dirofilariasis control measurements.[3] Such screening can be achieved by the detection of microfilariae, antigen testing (commercial kits available only for *D immitis*), or by molecular biology methods. Microfilaria screening may not be as sensitive as antigen testing, as shown in a study in which 20% of dogs were falsely negative.[1] However, microfilaria observation is important, not only because it validates serologic test results but also because it identifies dogs serving as a reservoir, thus alerting veterinarians to potential adverse reactions when using microfilaricide drugs.[4] Currently, 2 methods are recommended for microfilaria detection: membrane filtration and the modified Knott's test (MKT).[5] The latter is the most commonly used, being considered highly sensitive and specific.[5,6] Nevertheless, to the best of our knowledge, only a single study compared it with the buffy coat smear (BCS).[7] The BCS has been accepted for many years for the diagnosis of human filariasis[8], but veterinary studies have exclusively focused on the direct observation of the (unbroken) microhematocrit tube at low magnification [7,9,10], disregarding the use of BCS. Direct observation of hematocrit preparations proved to be useful in microfilaria screening, but has inherent limitations in species identification, as morphologic details are difficult to assess.[11]

The main purpose of this study was to compare the MKT with the BCS for the detection of microfilariae, and to evaluate its applicability under field study conditions.

Materials and Methods

Following a first report of dirofilariasis in Maio Island, Republic of Cabo Verde[12], we conducted a prospective study and screened about 30% of the canine population on that island[13], in July 2012. After a clinical examination including verification of the dogs identification, 2 mL of blood were collected by jugular venipuncture and placed in EDTA tubes (2.5 mL, K3 EDTA; FL medical, Torreglia, Italy), stored at +4°C until processed (always < 12 h), and used for performing BCS and MKT. In order to avoid biologic periodicity of microfilarial release to blood circulation, samples were collected between 16:00 and 18:00 (4:00 and 6:00 pm).

For BCS, plain capillary tubes were filled to 80% of their length with EDTA blood (approx. 65 µL), plugged with clay at one end placed in a microhematocrit centrifuge (International Equipment Co., IEC MB Centrifuge, Needham, MA, USA), and centrifuged at 12,700g for 5 min. Under field conditions, the centrifuge was directly connected to a field vehicle battery using an Enercell 350 W High Power Inverter for electricity supply. Tubes were then cut at the plasma–buffy coat interface with a glass cutter knife, and a paper clip was used to push the clay plug so that the buffy coat and one drop of plasma were placed on a glass slide for smear preparation (by edge of slide). Air-dried smears were stained with Diff Quik (Hemacolor, Merck, Germany).

For the MKT, 1 mL of EDTA blood was added to 9 mL of 2% buffered formalin in a glass centrifuge tube and thoroughly mixed by inverting the tube several times. After centrifugation (International Equipment Co., IEC 57154H) at 1800g for 5 min, the supernatant was decanted and a drop of 0.1% aqueous methylene blue was mixed with the sediment. Then, a drop of the mixture was placed on a slide and covered with a cover slip.

Slides obtained by both methods were screened by light microscopy at 200x and 400 x magnification, and results of MKT were evaluated without knowledge of the outcome of the other test method. In BCS, we recorded the number of microfilariae per slide, in order to estimate the

parasite load, and microfilariae were measured in microscopic images (1000 x magnifica- tion) using ImageJ software (imagej.net) for identification of the Dirofilaria species.[14] The morphologic identification was later confirmed by a direct polymerase chain reaction (PCR) assay, as detailed elsewhere.[15]

The diagnostic sensitivity (ie, the probability of MKT and BCS to identify microfilariae in infected dogs) was compared using the McNemar statistical test. In addition, we estimated the proportion of agreement, the Kappa, the prevalence adjusted bias adjusted kappa (PABAK), the Chamberlain's percent positive agreement (PPA), and the bias index using the software SPSS18 (IBM, Armonk, NY, USA).[16,17]

Results

The 150 dogs (101 males and 49 females) were sampled in rural and urban areas (approximately 50% each). In this population, the 2 tests rendered similar results, the prevalence with the MKT was 4.67%, and with the BCS it was 5.33% (Table 1). Both tests had the same ability to screen for microfilariae, without statistical differences in the McNemar test (P = 1).

Table 1. Comparison between modified Knott's test (MKT) and buffy coat smear (BCS) in the detection of microfilariae in dogs on Maio Island (Republic of Cabo Verde).

	MKT		
	Positive	Negative	Total
BCS			
Positive	7	1	8
Negative	0	142	142
Total	7	143	150

The number of microfilariae per BCS ranged from 1 to 12 per 65 µL of blood, resulting in an estimated parasite load of 15 to 185 microfilariae/mL. All samples negative in the BCS were also negative in the MKT. The proportion of agreement was 0.99, the Cohen's kappa coefficient was 0.930 (95% confidence interval: 0.793–1), while the PABAK was 0.987 and the PPA was 0.875. The bias index was 0.007. These values correspond to an almost perfect agreement between the 2 tests.[16]

The morphology of the microfilariae was compatible with *Dirofilaria repens* (Figure 1). The mean width was 6.66 ± 0.56 µm, and the mean length 267.1 ± 19.7 µm (total length). Microfilariae had a typical short (1.42 ± 0.26 µm) cephalic space anterior to the 2 nuclei.[14] The tail measured 19.1 ± 4.4 µm and was sometimes difficult to see due to overlay by erythrocytes. On occasion an umbrella handle shape was observed. The morphologic identification of *Dirofilaria repens* was confirmed by PCR[15] in all positive cases. No *Dirofilaria immitis* microfilariae were found (data not shown).

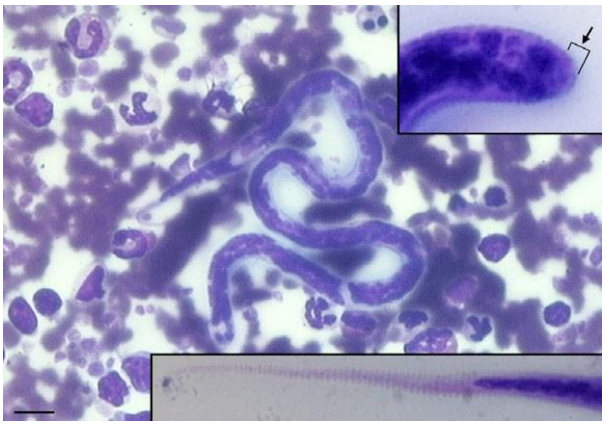

Figure 1. Microfilaria of *Dirofilaria repens* in a buffy coat smear of an infected dog. Note the typical short clear area in the cephalic end (arrow in the upper inset). The tail is filiform and rarely ends as an umbrella handle shape (lower inset); bar = 10 μm.

Discussion

This study confirms that canine dirofilariasis exists on Maio Island, Republic of Cabo Verde, but as opposed to a previous report[12], where *Dirofilaria immitis* was identified—this is a new finding as this species has never been reported in West Africa in contrast to South Africa.[1] Moreover, it shows a different pattern regarding Atlantic islands, namely Madeira and Canary Islands, where only *Dirofilaria immitis* has been reported and at prevalence rates of 30% or higher.[1]

The comparable diagnostic performance between MKT and BCS, the main focus of this technical report, is in line with previous findings.[7,9,10] However, previous studies[7,10] compared MKT with the direct observation of (unbroken) hematocrit tubes under the microscope, and not BCS as in this report. So far, BCS was described in only one previous study, where it was applied in cases that were negative on direct microscopic observation of the tube.[7] According to this author, the BCS increased the diagnostic yield from 10% to 30%, having 100% diagnostic sensitivity in cases with an infestation of > 25 microfilariae/mL.[7] Important advantages of BCS over the direct observation of (unbroken) tubes include the morphologic evaluation of microfilariae allowing species differentiation, and the diagnosis of animals infested with low numbers. Moreover, the BCS permits a simultaneous screen for other hemoparasites (eg, *Hepatozoon, Ehrlichia, Anaplasma*)[18], which is of paramount importance in field studies where several different parasites are often observed. Finally, it results in air-fixed definitive preparations that can be stored for later staining and analysis. It is noteworthy that previously a mixture of 1:20 methylene blue/formalin was used to stain the BCS.[7] In our experience Diff Quik staining provides more morphologic detail and moreover, those authors used QBC tubes that are much more expensive than plain capillary tubes (approximately 4.5$ vs 9¢ per tube).

In this study, we assessed test agreement by various parameters (proportion of agreement, Kappa, PABAK, PPA), as recommended.[16,17] There is a consensus that the kappa coefficient alone is only sufficient when the diagonals of a cross-tabulation table are fairly balanced, being ambiguous when the prevalence of a given response or result is very high or low. As this occurred in our case, we used PABAK to adjust the values, showing a slight improvement in the kappa coefficient. Even though this adjustment is widely used[16], it has been argued that it is a linear function of the agreement proportion and that instead of PABAK, PPA should be used when the prevalence is very high or low.[17] Whatever the case, all those coefficients pointed to an almost perfect agreement between MKT and BCS, and the McNemar statistical test further showed that both methods were of comparable diagnostic sen- sitivity in detecting microfilaria. Nevertheless, the latter is preferable for field studies for several reasons: (1) no formalin is used (besides being toxic it is often difficult to transport to remote locations); (2) less blood is necessary—this can be relevant for small dogs or

for assessing microfilariae in small species, like birds, ferrets, or lizards; (3) plasma is separated and can be used for protein assessment; and (4) other hemoparasites can be screened for. Regarding the negative MKT result in a dog which was BCS microfilaria positive (Table 1), it was probably due to the low number of microfilariae in circulation. The MKT usually involves evaluation of a drop of sediment[5], but it has been shown that such a procedure can lead to sampling errors (and consequently to negative results) in dogs with < 25 microfilariae/mL, which can only be sur- passed by the use of the entire sediment.[7]

Regarding the morphometric data, the measurements in BCS were 25% less for length and width than the reported data for D. repens in MKT preparations.[6] This is probably due to the effect of air-drying and the presence of methanol, which tends to reduce the size of microfilariae, compared with the MKT.[6] Moreover, microfilariae in BCS also appear shorter than in air- dried (thin) blood smears stained with Giemsa[14]; nevertheless, they keep a small free cephalic space 3-fold shorter than that of *D immitis* (data not shown) that allows an easy recognition of the species.[14]

In conclusion, our results indicate that the BCS has high diagnostic sensitivity, being reliable for the detection and speciation of microfilaria. As it is technically easier and less cumbersome, it could replace the MKT not only in the everyday practice[7] but also in large studies devoted to dirofilariasis detection in remote places.

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