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Comparative methodology for the detection and differentiation of circulating microfilariae of *Dirofilaria immitis* in the dog

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Abstract

The sensitivities of the Knott's test (four 20-μl sediment aliquots), quantitative buffy coat capillary tube method (QBC tube, 111 μl of whole blood) and direct blood smear (DBS, 20 μl of whole blood) were evaluated for the detection of microfilaraemia in dogs. Undiluted whole blood samples taken from 70 *Dirofilaria immitis* antigen-positive dogs and 10 serially diluted microfilaraemic blood samples at concentrations of 400, 200, 100, 50, 25 and 12 microfilariae (mff) ml^{-1} were examined. For filarial speciation, the buffy coat of QBC tubes was mixed with one drop of methylene blue–formalin solution and examined as a direct smear. In 52/70 microfilaraemic blood samples, the number of mff ranged from 12 to 321987 ml^{-1} (median: 3199 ml^{-1}). The diagnostic sensitivity of the Knott's test, QBC tube method and DBS in undiluted blood samples attained the 100%, 98% and 92.3% levels, respectively. Eighteen dogs tested amicrofilaraemic by all three methods. At concentrations of 400 mff ml^{-1}, a 100% sensitivity was found by all three methods, while at 200 mff ml^{-1} the Knott's test, QBC tube and DBS were 100%, 100% and 90% sensitive, respectively. The relevant figures at 100 mff ml^{-1} were 100%, 100% and 80%, at 50 mff ml^{-1} 100%, 100% and 50%, at 25 mff ml^{-1} 100%, 100% and 10% and at 12 mff ml^{-1} 80%, 50% and 10%. At 50 and 25 mff ml^{-1}, the DBS was less sensitive compared to the other two methods, while at 12 mff ml^{-1}, only to the Knott's test. A significant correlation was found between the QBC tube method and Knott's test regarding mff speciation. Therefore, the QBC method may be considered a reliable alternative to the Knott's test for both the detection and speciation of mff in the dog.

Introduction

At the present time, the detection of *Dirofilaria immitis* microfilaraemia is considered supplemental to antigen testing in the diagnosis of canine heartworm disease (Knight, 1998). Also, it may be of value in the assessment

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of potential risks associated with the microfilaricidal treatment or chemoprophylaxis in dogs with large microfilarial loads (Knight, 1998; Dillon, 2000). Concentration methods (Knott's and filter test) have consistently been more sensitive compared to direct blood smears (DBS) and haematocrit capillary tube (QBC tube method) in both detecting microfilariae (mff), and differentiating them to species level (Wylie, 1970; Rawlings, 1986). Nevertheless, the time- and cost-effectiveness of the latter two methods make them attractive alternatives in everyday practice. Only limited information has been available on the blood volume required in DBS, and most importantly, the lowest mff concentration permitting a quick detection with the DBS and QBC tube methods. The sensitivity of DBS has been reported at a high level only when mff counts exceeded 1000 ml^{-1} (Rawlings, 1986), but in a recent study a 100% sensitivity was documented for mff counts higher than 50 ml^{-1} (Courtney & Zeng, 2001). In DBS the differentiation between *D. immitis* and *Dipetalonema reconditum* mff can be assessed by their number and motility pattern, though this method is considered of low diagnostic yield, especially for those areas where *D. immitis* frequently coexists with other non-pathogenic filarial species (Papazahariadou *et al.*, 1994; Dillon, 2000). Brown & Barsandi (1988) claimed a 100% sensitivity with the QBC tube for mff counts as low as 160 ml^{-1}, while their visualization has been considered excellent in another study (Levine *et al.*, 1986). However, no attempt was made to differentiate *D. immitis* mff from those of *D. reconditum* in either of these studies.

In the present study, the sensitivities of the modified Knott's test (four 20-μl sediment aliquots), DBS (20 μl of whole blood) and QBC tube method (111 μl of whole blood) were compared for the detection of mff in both undiluted and serially diluted blood samples, obtained from *D. immitis* antigen-positive dogs. The correlation between Knott's test and the QBC tube method for filarial speciation was also investigated.

Materials and methods

Blood samples

Seventy *D. immitis* antigen-positive dogs (Snap® Canine Heartworm PF, IDDEX) of various breeds, including 37 males and 33 females, with an age range of 1 to 11 years (median: 4.5 years), admitted to the Clinic of Companion Animal Medicine, Aristotle University of Thessaloniki for various medical reasons, were used in the study. In all the dogs, whole blood samples were examined for mff with the Knott's test, QBC tube method and DBS. In each dog, the total number of mff ml^{-1}, irrespective of their species, was counted by examining the whole sediment in the Knott's test, where a total of 1 ml of whole blood was used. To further evaluate the sensitivity of the three methods in low level microfilaraemia, ten blood samples taken from microfilaraemic dogs were serially diluted with the blood obtained from a clinically healthy and amicrofilaraemic dog until concentrations of 400 ml^{-1}, 200 ml^{-1}, 100 ml^{-1}, 50 ml^{-1}, 25 ml^{-1} and 12 mff ml^{-1} were achieved. The accuracy of the dilution procedure was confirmed by the Knott's test.

Testing for microfilariae

The modified Knott's test (four 20-μl sediment aliquots) (Kelly, 1973), DBS (20 μl of fresh whole blood under a cover slip) and QBC tube method (111 μl of whole blood) (Brown & Barsanti, 1988) were tested against undiluted (n = 70) and serially diluted (n = 10) blood samples. The QBC tube was mounted on a glass slide with the use of adhesive material to permit thorough visualization. For definitive evaluation of negative results as well as for filarial species differentiation, the QBC tube was gently broken at the upper-most level of the plastic float, which was removed, to allow one drop of a methylene blue (1:1000) and formalin (2%) 1:20 mixture to cover the buffy coat with the aid of a 25-G needle attached to a 1-ml syringe. The stained buffy coat was subsequently pipetted off and examined as a direct smear under a cover slip. The detection of mff by all three methods, and filarial differentiation by the Knott's test and QBC tube method were carried out by two independent examiners, unaware of the microfilarial status of the animals, using an ocular micrometer-equipped microscope, at × 100 and × 400 magnification, respectively. The identification of filarial species was based on well established morphological criteria (Lindsey, 1965; Watson *et al.*, 1973).

Statistical analysis

McNemar's chi-square test (Statistical Analysis systems, 2000, SAS rel. SAS Insitute, Cary, North Carolina) was used in pairwise comparisons to determine the diagnostic sensitivity of the three methods in both undiluted and serially diluted blood samples. Any correlation between the number of *Dirofilaria repens* or *D. immitis* mff detected either by the Knott's test or QBC tube method was measured using a Spearman's rank-order correlation coefficient. The significance was evaluated at the 5% level.

Results

Fifty-two of 70 (74.3%) dogs tested microfilaraemic, with circulating mff ranging from 12 to 321987 ml^{-1} (median: 3199 ml^{-1}). The number of dogs harbouring from 1 to 400 mff ml^{-1} was 7/52 (13.5%), while 45/52 (86.5%) harboured more than 400 mff ml^{-1}. Eighteen dogs (25.7%) were found to be amicrofilaraemic by all three methods. Single-species microfilaraemia with *D. immitis* (25/52 – 48%) and *D. repens* (4/52 – 7.6%), and double-species microfilaraemia with *D. immitis*/*D. repens* (21/52 – 40.3%) and *D. immitis*/*D. reconditum* (2/52 – 3.8%), were demonstrated.

In undiluted blood samples of microfilaraemic dogs (n = 52), the diagnostic sensitivity of the Knott's test, QBC tube method and DBS was 100% (52/52), 98% (51/52) and 92.3% (48/52), respectively, but no significance was found at any level. In the QBC, negative results were obtained in one sample containing 12 mff ml^{-1}, and in DBS four smears contained 12, 35, 133 and 3726 mff ml^{-1}. The sensitivity of the three methods in serially diluted blood samples is shown in fig. 1. Differences in the sensitivity, for all serial mff concentrations, between the QBC tube method and Knott's test were not significant.

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At 50 mff ml^{-1}, the DBS was less sensitive compared with the other two methods, but these differences were not significant ($P = 0.062$), in contrast to the 25 mff ml^{-1} concentration ($P = 0.004$). At 12 mff ml^{-1}, the DBS was less sensitive only to the Knott's test ($P = 0.015$), while no difference was found between the DBS and QBC methods ($P = 0.125$) or between the QBC method and the Knott's test ($P = 0.25$). The time required to identify mff ranged from a few seconds to 10 minutes, and was dependent on their concentrations, but not on the method applied. In the QBC, three diluted blood samples with 12, one with 25 and one with 50 mff ml^{-1} were found to be positive only after breaking the upper part of the tube. No morphological changes were seen in the QBC tube-processed mff. The Spearman's rank-order correlation coefficient between the Knott's test and QBC for *D. immitis* was 0.79 (95% CI: 0.64–0.93, $P < 0.0001$) and for *D. repens* 0.70 (95% CI: 0.49–0.92, $P < 0.0001$). *Dipetalonema reconditum* was not evaluated because there were only two dogs detected by the Knott's test and none with the QBC method.

Discussion

In the present study, the mean number of mff (3199 ml^{-1}), was well within the sensitivity limit of all three methods (Rawlings, 1986; Brown & Barsanti, 1988; Courtney & Zeng, 2001). Since microfilaraemia of less than 400 ml^{-1} was detected only in seven dogs, serially diluted blood samples also needed to be examined to evaluate the sensitivity of the three methods at low level microfilaraemia.

The sensitivity of a 20-μl DBS was almost 100% for mff counts as low as 400 ml^{-1}, a higher figure than that previously reported (Rawlings, 1986), but lower than in the concentration of 50 ml^{-1} (Courtney & Zeng, 2001). The larger blood volume (50 μl) used by Courtney & Zeng (2001), could explain the difference. Both studies documented the sensitivity of the DBS in detecting microfilaraemia at much lower mff concentrations than those reportedly associated with the appearance of adverse reactions following the inadvertent administration of microfilaricidal medication. Hypersensitivity reactions have been reported in dogs harbouring 500 mff ml^{-1} of peripheral blood following diethylcarbamazine administration (Palumbo *et al.*, 1981), while with macrolides such as ivermectin and milbemycin oxime, these reactions may occur with mff numbers exceeding 5000 (Knight, 1998), or 40000 ml^{-1} (Dillon, 2000).

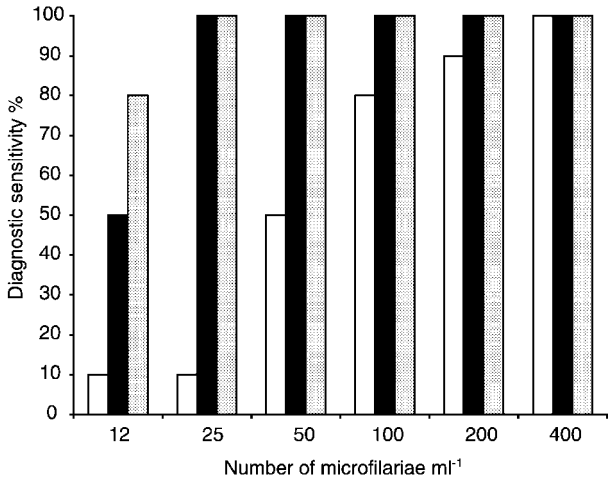

Fig. 1. Diagnostic sensitivity of the direct blood smear (□), quantitative buffy coat tube method (■) and modified Knott's test (▤) in ten serially diluted blood samples taken from an equal number of microfilaraemic dogs infected with *Dirofilaria immitis*.

The QBC tube method appeared to exceed the sensitivity of the DBS and approached that of the Knott's test even with mff counts as low as 12 ml^{-1}. The 100% sensitivity at 25 mff ml^{-1}, is considerably higher than at 160 mff ml^{-1} (Brown & Barsanti, 1988), but these authors used only a limited visualization time (60 seconds). Another explanation could be mff motility in the capillary tube lasting approximately 20 minutes subsequent to blood sampling (Kelly, 1973). Therefore, any delay in the process may suppress mff motility, and reduce the sensitivity of the method. This problem may be overcome by breaking-up the QBC tube and reexamining the BC under the microscope, whenever no filarial motility is observed. In the present study, a total of five QBC tubes initially reported negative results, which were subsequently found to be positive. Other investigators have also claimed the convenience of the QBC tube method for routine filarial screening, although there was no reference to the lowest possible concentrations of mff for a quick and reliable detection (Wylie, 1970; Levine *et al.*, 1986; Wang, 1998).

To our knowledge, this is the first attempt to accomplish a reliable filarial speciation in the dog with the aid of the QBC tube. In human filariasis, the QBC method has also been successfully used for filarial differentiation, but with the aid of fluorescent microscopy (Long *et al.*, 1990; Freedman & Berry, 1992; Wang, 1998). The aforementioned technique could also be applied successfully, despite the fact that the Knott's test is considered the gold standard for detecting and differentiating mff in the peripheral blood of the dog (Rawlings, 1986). The somatic distribution of acid phosphatase activity may further confirm mff differentiation in canine blood, but a good correlation has also been reported between acid phosphatase activity and the Knott's test (Gringoli *et al.*, 2001). The use of four 20-μl preparations in the Knott's test resulted in 100% sensitivity in all but two blood samples containing 12 mff ml^{-1}. The screening of the entire sediment would overcome this problem but time allowances in a busy small animal practice would make a thorough examination of the entire sediment somewhat unrealistic (Kelly, 1973; Watson *et al.*, 1973). No attempt has been made to correlate mff numbers or motility patterns with their speciation in both QBC tubes and DBS, because of the misleading results obtained in our experience. This could be attributed to the high prevalence of *D. repens* infection in the Greek canine population, either as a single entity or concurrently with *D. immitis* (Papazahariadou *et al.*, 1994).

In conclusion, these results indicate that the QBC tube method is sensitive and reliable for the detection and speciation of mff in the dog to such a degree that it may substitute the Knott's test in everyday practice.

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