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Research paper

Acetic acid as an alternative reagent in the modified Knott test

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ABSTRACT

The suitability of acetic acid as a safer alternative to formalin in the modified Knott test was evaluated for the diagnosis of canine heartworm (*Dirofilaria immitis*). Microfilaria concentration was measured by both methods and found to agree within reasonable limits (−5.84 % bias; −88.1–76.4 % limits of agreement). The level of agreement was lower when samples were prepared with a 24 h delay, but this was due to the formalin method tending to yield lower counts (−20.1 % bias; −90.5–50.2 % limits of agreement). Clearing the sample of hemoglobin improves readability and is a key feature of the modified Knott test. Hemolysis was significantly lower in the acetic acid method than the formalin method as measured by red blood cell count (6.83×10^6 and 8.79×10^6 cells/ml, respectively; $p = 0.015$) and absorbance at 415 nm (33.20 and 34.75, respectively; $p < 0.001$). Visual assessment, however, revealed little practical difference in readability. Finally, lengths of microfilariae were measured to ensure the validity of species identification by the acetic acid method; mean length was significantly shorter after acetic acid treatment (273 μm) than formalin treatment (316 μm; $p < 0.001$). Length reduction was also observed in acetic acid-treated *Acanthocheilonema reconditum* (254 μm versus 262 μm; $p = 0.035$), though these samples were stored prior to testing and are not directly comparable. We conclude that, while the readability of samples is similar for both methods, species differentiation must still be accomplished by other means. For most clinical purposes in determining the presence or absence of blood circulating microfilariae, however, acetic acid appears to be a suitable alternative to formalin in the modified Knott test.

1. Introduction

Although antigen testing is the most sensitive method for diagnosing canine heartworm (*Dirofilaria immitis*) and is useful in detecting both prepatent and occult infections, microfilaria (mf) testing remains relevant, and the American Heartworm Society recommends its use in conjunction with serological methods(American Heartworm Society, 2018). This is in part due to antigen-negative/mf-positive cases, which are believed to result from antigen-antibody complex formation; one study reports the rate of these false negatives at 7.1 % (Velasquez et al., 2014). The other persistent incentive to diagnose microfilaremia is to mitigate adverse events brought on by microfilaricidal treatment, which are reported to occur in animals with higher mf concentrations (Neer and Hoskins, 1989).

Low numbers of mf can be missed in direct blood smears or with the capillary tube (QBC) method, therefore concentration methods are preferred for their sensitivity. For filtration methods, like the Difil test (Tilley and Wilkins, 1974), high mf concentrations can obstruct membranes, and the present lack of commercially available kits demands more effort on the part of the clinician. The modified Knott test (Knott, 1939) is preferred and is the recommended test for circulating mf (American Heartworm Society, 2018). It also allows morphology-based species identification, which is especially useful in regions where infection with *Acanthocheilonema reconditum* occurs (Valenciano et al., 2014). This technique involves diluting 1 ml blood in 9–10 ml formalin (2 %), which lyses red blood cells to improve readability and preserves mf for deferred examinations. Formalin, however, is a known carcinogen that requires special precautions in handling, storage, and disposal (National Toxicology Program, 2011). For all its benefits, re-evaluating the use of formalin in the modified Knott test may yield safer alternatives.

Like formalin, acetic acid possesses hemolytic and preservative properties; it is useful as a fixative for some laboratory applications and is the key hemolytic reagent in Türk's solution for leukocyte quantification in whole blood (Bernadette F. Rodak et al., 2007; Leong, 1994). Unlike formalin, however, it is non-carcinogenic, and is readily

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available at sufficient concentrations in the form of household vinegar. For these reasons we sought to investigate the suitability of acetic acid as an alternative to formalin in the modified Knott test for canine heartworm diagnosis. We hypothesize that the two reagents will perform similarly with respect to all key functions of the test: mf count, short-term preservation of mf, red blood cell lysis, and maintenance of morphological integrity for species identification.

2. Materials and methods

2.1. Parasites

Microfilaremic venous blood was obtained from six beagle dogs previously infected with *D. immitis* (2005 MO or JYD-27 isolate) via subcutaneous injection of third-stage larvae. Animals were maintained by the FR3 (Filariasis Research Reagent Resource Center, Athens, GA). Microfilaremic blood containing *A. reconditum* microfilariae was obtained from the University of Florida, College of Veterinary Medicine (Gainesville, FL); these samples were maintained at 4 °C for at least one week prior to use in the present study.

2.2. Modified Knott test

Venous blood samples were collected in evacuated sodium heparin tubes. The modified Knott test was performed within 2 h of collection according to either standard procedures, utilizing formalin, or our modification, utilizing acetic acid. In a 15-ml conical tube, 10 ml of either 2 % formalin or 2 % acetic acid was added to 1 ml blood; the formalin solution was diluted from phosphate buffered formalin (10 %), while the acetic acid solution was diluted from distilled household vinegar (5 %), both in deionized water. The tube was inverted at least five times to mix and centrifuged for 10 min at 650 rcf. The supernatant was removed by decanting and two drops methylene blue (approximately 100 µl) were added. The pellet was mixed by pipetting.

2.3. Microfilaria counts

The pellet obtained from the modified Knott test was dispensed in 20 µl aliquots onto three slides per sample, and coverslipped. To calculate concentration, the total volume of the pellet mixture was determined by pipetting. Slides were examined under a compound microscope (Nikon Eclipse E200, Nikon Corporation, Japan) at 100× magnification with blinding to treatment group. To evaluate mf preservation, this procedure was repeated with another three aliquots from the pellet 24 h later. Giemsa-stained thick smears were prepared according to standard procedures (Eberhard and Lammie, 1991) as a gold standard against which mf counts could be evaluated.

2.4. Hemolysis evaluation

A hemocytometer (Reichert Bright-Line™) was used to assess the lysis of red blood cells by both reagents. For each of three study animals, 20 µl samples were taken from the stained pellet obtained by each method (n = 3). Five squares of the finely gridded section (20 nl total) were used to calculate cell count per sample with blinding to treatment group.

The supernatant of each sample obtained above was also evaluated by the Harboe direct spectrophotometric method for free hemoglobin. Samples were diluted 1/10 in deionized water and analyzed with a SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA), measuring absorbance at 415 nm, near the peak absorbance wavelength for oxyhemoglobin (Han et al., 2010).

2.5. Microfilaria morphology

The lengths of mf obtained through each modified Knott test method (n = 9 per method) were measured using an eyepiece micrometer and inverted compound microscope (Nikon TMS, Nikon Corporation, Japan) at 400× magnification with blinding to treatment group. Mf were obtained from three tests performed with samples from each of three study animals and examined on the day of collection. *A. reconditum* samples were equilibrated to room temperature prior to treatment and measurement (n = 9 per method).

2.6. Statistical analysis

Agreement of mf count between the two testing protocols was assessed by linear regression and Bland-Altman analysis with 95 % confidence interval limits of agreement (Bland and Altman, 1986). Paired, one-tailed Student's *t*-tests were performed for cell count and absorbance data (paired by blood sample) to detect any decrease in mean hemolysis in the acetic acid method relative to the standard method. A paired, two-tailed Student's *t*-test was performed to detect differences in the mean length of mf (paired by blood sample) obtained via each method. Analyses were performed using GraphPad Prism® version 6 (GraphPad Software, Inc., San Diego, CA).

3. Results

A clear correlation can be seen between mf counts obtained via the modified Knott test using formalin and acetic acid solutions on both the day of sample collection and the following day, with R^2 values of 0.964 and 0.982, respectively (Fig. 1). Bland-Altman plots are presented to demonstrate agreement between the two methods at both timepoints. The formalin-based test tended to yield lower mf counts than the acetic acid modification on the day of collection (−5.84 %; −88.1–76.4% limits of agreement) and even more markedly the following day (−20.1 %; −90.5–50.2% limits of agreement). This was similar to the agreement between the formalin-based test and the thick smear gold standard on the day of collection (−6.66 %; −71.0–57.7% limits of agreement) and 24 h later (−21.6 %; −62.0–18.8% limits of agreement).

Hemolysis, as measured by the mean concentration of non-lysed red blood cells in the modified Knott test product, was less pronounced in the acetic acid method ($8.79 \times 10^6 \pm 1.47 \times 10^6$ cells/ml) than in the formalin method ($6.83 \times 10^6 \pm 2.23 \times 10^6$ cells/ml), and this difference was calculated to be significant ($p = 0.015$; Fig. 2a). Measuring hemolysis by the absorbance of free hemoglobin produced similar results, with the acetic acid supernatant demonstrating a lower mean absorbance (33.20 ± 0.334) compared to the formalin supernatant (34.75 ± 0.960), which was also calculated to be significant ($p < 0.001$; Fig. 2b).

The mean length of *D. immitis* mf exposed to acetic acid treatment was calculated to be 273 µm (SD = 7.88 µm), significantly shorter than that of mf exposed to the formalin-based test, which averaged 316 µm (SD = 4.42 µm; $p < 0.001$; Fig. 3a). The mean length of *A. reconditum* mf was also observed to differ with acetic acid treatment (254 ± 4.60 µm) and formalin treatment (262 ± 9.38 µm), and, while less pronounced, this difference was significant ($p = 0.035$; Fig. 3b).

4. Discussion

Testing for the presence of circulating microfilariae continues to be integral to the treatment and prevention of canine heartworm infection, both by complementing serological methods with imperfect sensitivity and by recognizing hazardous parasite levels before microfilaricidal therapy is administered. For these reasons, the American Heartworm Society guidelines advocate mf testing on all dogs (American Heartworm Society, 2018). However, concerns over exposure to formalin, a key reagent of the recommended modified Knott test, have prompted researchers and clinicians to explore safer alternatives. This need was only reinforced when the National Toxicology Program

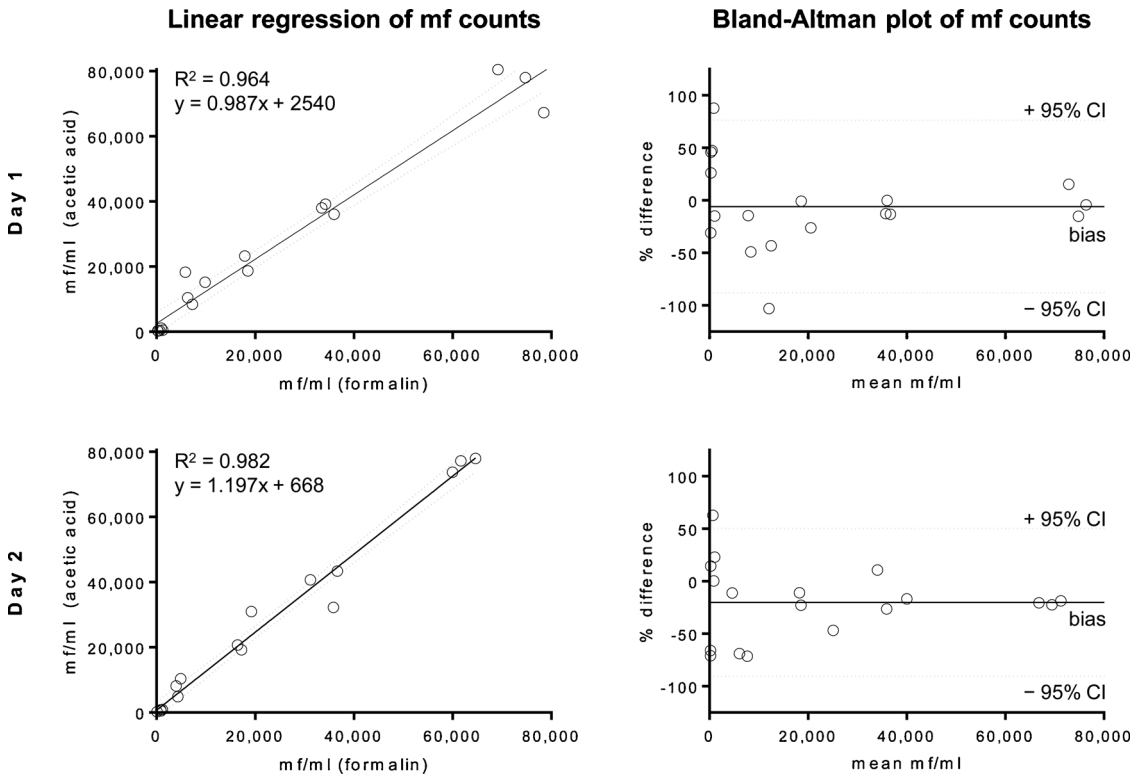

Fig. 1. Calculated microfilaria concentrations obtained by modified Knott tests using formalin and acetic acid. (a) linear regression with 95 % confidence intervals (dotted lines) with correlation and regression equations presented. (b) Bland-Altman plots of differences between concentrations by each method (formalin − acetic acid), with solid and dotted lines denoting bias and 95 % limits of agreement, respectively. Samples were prepared both on the day of collection (Day 1) and the following day (Day 2; n = 18).

reclassified formaldehyde as a known human carcinogen (National Toxicology Program, 2011). For these reasons, we evaluated acetic acid as a safer alternative to formalin in the modified Knott test.

We first assessed the suitability of both methods for enumerating mf. Visualizing parasites in blood smears is critical to such microscopic tests, and the modified Knott test requires formalin for the dual purposes of producing clear, readable samples and preserving the integrity of these samples for deferred reading. Unsurprisingly, mf concentrations calculated from each method correlated well. The agreement between the two methods, as represented by Bland-Altman plots (Fig. 1), is more revealing, and shows a slight bias of the standard, formalin-based method to yield lower counts than the acetic acid method (-5.84 %). This could be attributed to the occasional aggregation of mf observed in the test, which can obscure individual mf. We noted, however, that this phenomenon occurred less often with acetic acid treatment, possibly improving mf clarity. Parasite counts were obtained the following day to assess the short-term preservation of samples, demonstrating an even greater negative bias of the formalin method (-20.1 %). These findings are similar to the agreement biases we observed between the formalin-based modified Knott test and the Giemsa-stained thick

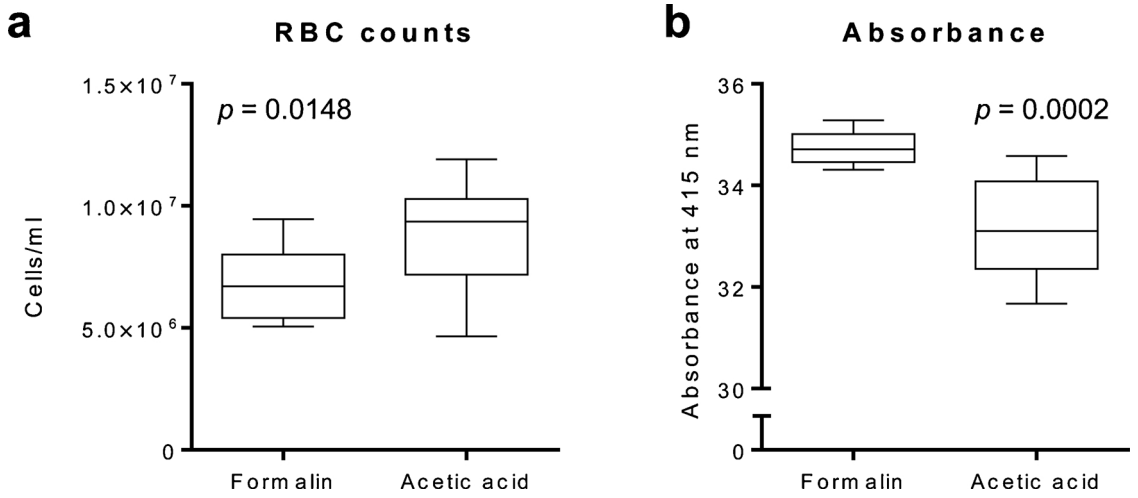

Fig. 2. Hemolytic activities of modified Knott tests using formalin and acetic acid. Hemolysis as measured by non-lysed red blood cell concentration (a) and absorbance at 415 nm (b) are presented as box and whisker plots. The boxes indicate the first and third quartiles while the whiskers indicate the minimum and maximum values of all sample means (n = 9) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

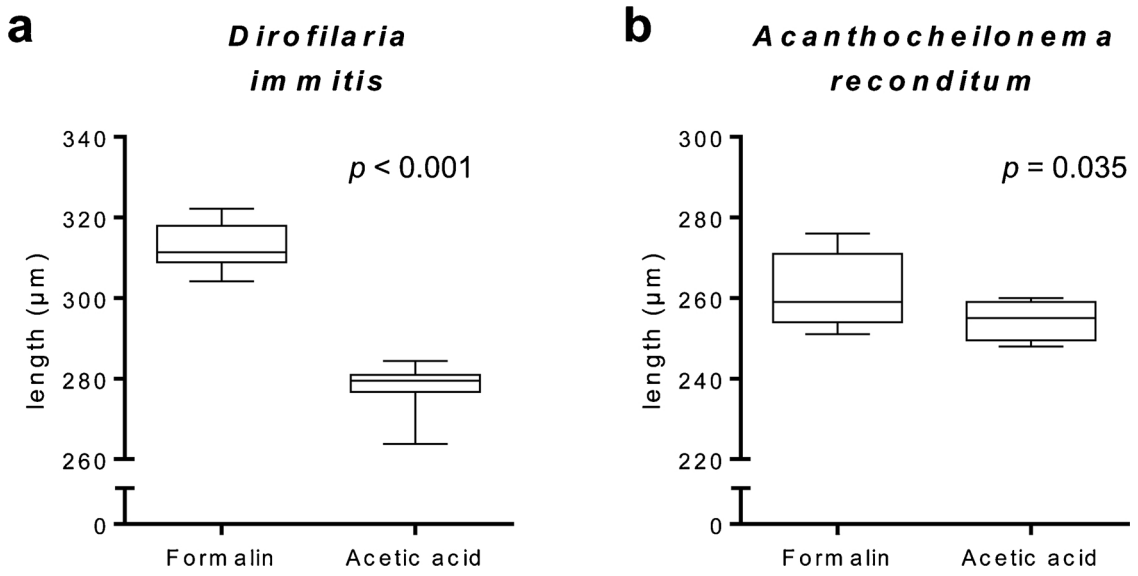

Fig. 3. Length of microfilaria (mf) obtained by modified Knott tests using formalin and acetic acid. Mf lengths in *Dirofilaria immitis* (a) and *Acanthocheilonema reconditum* (b) samples from each method are presented as box and whisker plots. The boxes indicate the first and third quartiles while the whiskers indicate the minimum and maximum values of all sample means (n = 9).

smear, the gold standard for mf quantification. All concentration techniques are semi-quantitative at best, and noting the biases observed against the gold standard, we consider the use of acetic acid in the modified Knott test to produce clinically similar counts and that samples are preserved similarly well over the timeframe tested.

Like other filarial species, *D. immitis* exhibits a circadian microfilarial periodicity, in which the concentration in peripheral blood rises and falls throughout the day; this peak often, but not always, occurs in the late afternoon (Church et al., 1976; Ranjbar-Bahadori et al., 2011; Rhee et al., 1998). While blood collection could be timed to occur with peak microfilaremia, mf concentration techniques, like the modified Knott test, were developed to detect circulating mf at any time of day, which is especially helpful in lymphatic filariasis of humans that may otherwise be undetectable during daylight hours (Knott, 1939). Considering this sensitivity over direct blood smears, we expect the acetic acid-based test to perform as a reliable measure of microfilaremia status, on par with the formalin method.

Hemolysis is a key benefit of the modified Knott test, resulting from the hypotonicity of 2 % formalin. This improves the readability of slides by removing a large fraction of red blood cells from the sample. We examined hemolysis by two methods: comparing concentrations of non-lysed red blood cells and comparing the absorbance of hemoglobin released by hemolysis. Any increase in hemolytic activity is only expected to improve readability, thus our analysis only tested for significant reductions. In both methods, however, we observed lower levels of hemolysis in the acetic acid modified Knott test than in the standard test, and these differences were statistically significant (Fig. 2). Despite the clear differences obtained through each method, the clarity of formalin- and acetic acid-derived samples appear visually similar when blindly evaluated, leading us to question whether these findings bear any clinical significance. The only consistent difference observed microscopically in the quality of samples was a tendency for slightly more stain to be retained by remnants of lysed red blood cells in the formalin-based test than the acetic acid modification (Fig. 4).

Finally, the modified Knott test utilizing 2 % formalin is valuable in that it allows in-clinic, morphological differentiation between similar species; most notable of these is *A. reconditum*, which has worldwide distribution and, in some regions, is even more prevalent than *D. immitis* (Brianti et al., 2012). This parasite poses less of a pathogenic risk than heartworm, and so the two must be differentiated to avoid unnecessary treatment procedures. It is also possible to distinguish the mf of *D. repens*, a common parasite of dogs outside the Americas, on the basis of morphology, which may be important based on a clinic's location or animal travel history (Magnis et al., 2013). A key feature for differential diagnosis is mf length, thus we compared the length of mf obtained by each method to assess the suitability of the acetic acid modified Knott test for morphological identification. We observed significantly lower mean lengths in the acetic acid method than the formalin method (Fig. 3a). The mean length for acetic acid-treated mf (273 µm) lies outside the diagnostic range for *D. immitis* (295–325 µm), falling instead within the range for *A. reconditum* (250–288 µm; American Heartworm Society, 2018). The results obtained by this method were, therefore, not directly comparable to the conventional test and indicate that, unless new reference ranges are established, acetic acid treatment cannot be used for effective species identification. When the acetic acid method was performed on a sample of *A. reconditum*-infected blood, we observed a reduction in overall length compared to formalin-treated controls, and while this difference was significant, it was less pronounced than that observed with *D. immitis* (Fig. 3b). Furthermore, due to the limited availability of fresh *A. reconditum* samples, those available for the present study were stored by refrigeration prior to testing; while mf lengths were within the expected diagnostic range, this storage period in itself may have affected mf morphology, and further study is required to determine the effect of acetic acid on this parasite in freshly collected samples. With these caveats in mind, species identification can be accomplished by other means if *A. reconditum* is suspected. Besides the formalin-based modified Knott test, one such technique involves the preparation of direct blood smears (wet mounts) to observe motility; the mf of *A. reconditum* typically demonstrate progressive motility, readily crossing the field of view on examination, while *D. immitis* is characteristically nonprogressive. In addition, higher mf concentrations are generally associated with *D. immitis* infection compared to *A. reconditum* infection, and this can help direct a diagnosis (Valenciano et al., 2014). Radiography and echocardiography may also be useful in confirming mature heartworm infection (American Heartworm Society, 2018). While other morphological features (tail morphology in particular) appeared consistent with species descriptions, these differences alone may not be adequate for a reliable species identification. We therefore reason that, with the availability of common alternatives for species identification, the safety and consistency of the acetic acid-based test warrant consideration, provided this specific limitation is understood. If morphology-based

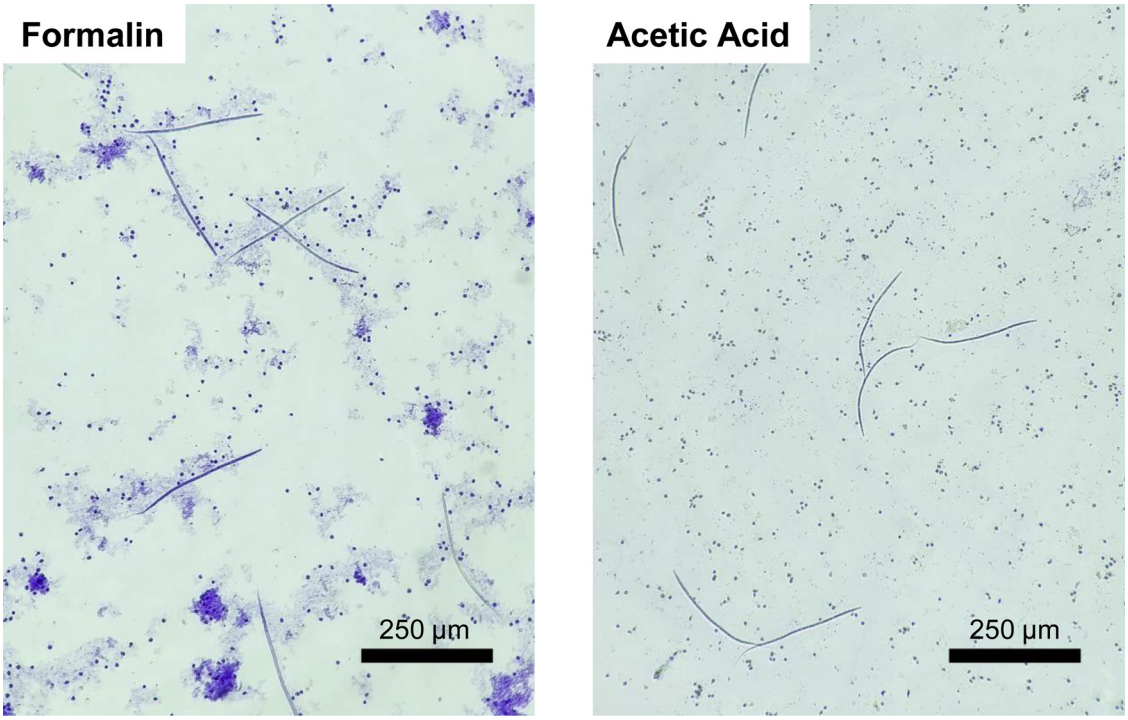

Fig. 4. Microfilariae obtained by modified Knott tests using (a) formalin and (b) acetic acid. Representative fields are shown, exhibiting the qualitative characteristics of samples prepared by each method. Photographs of mf were taken at 200× magnification using a Nikon Eclipse E200 compound microscope (Nikon Corporation, Japan).

identification of mf is desired, however, this must be done with formalin fixation to agree with established diagnostic reference ranges.

This work represents the first documented investigation of acetic acid as an alternative to formalin in the modified Knott test. There are clear benefits to a safer reagent, and especially one that is as readily available as household vinegar. Initial results suggested a similar output: mf count was comparable and, despite statistically significant differences in hemolysis by two measures, the readability of slides was considered subjectively similar. The lengths of mf, however, are significantly reduced after acetic acid treatment, likely due to more pronounced parasite dehydration. Our results thus far suggest that this reduction also occurs with *A. reconditum*, and further studies may establish diagnostic ranges for acetic acid treatment. While at present, this precludes morphology-based species differentiation, common alternatives exist to complement this limitation. We conclude, therefore, that for most clinical and research purposes, acetic acid appears to be a safe and suitable alternative to formalin in the modified Knott test.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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References

American Heartworm Society, 2018. Current Canine Guidelines for the Prevention. Diagnosis, and Management of Heartworm (*Dirofilaria immitis*) Infection in Dogs, Wilmington, DE. https://www.heartwormsociety.org/veterinary-resources/american-heartworm-society-guidelines.

Rodak, Bernadette F., Fritsma, George A., Doig, Kathryn, 2007. Hematology: Clinical Principles and Applications. Saunders, St. Louis, MO.

Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet (London, England) 1, 307–310.

Brianti, E., Gaglio, G., Napoli, E., Giannetto, S., Dantas-Torres, F., Bain, O., Otranto, D., 2012. New insights into the ecology and biology of *Acanthocheilonema reconditum* (Grassi, 1889) causing canine subcutaneous filariosis. Parasitology 139.

Church, E.M., Georgi, J.R., Robson, D.S., 1976. Analysis of the microfilarial periodicity of *Dirofilaria immitis*. Cornell Vet. 66, 333–346.

Eberhard, M.L., Lammie, P.J., 1991. Laboratory diagnosis of filariasis. Clin. Lab. Med. 11, 977–1010.

Han, V., Serrano, K., Devine, D.V., 2010. A comparative study of common techniques used to measure haemolysis in stored red cell concentrates. Vox Sang. 98, 116–123.

Knott, J., 1939. A method for making microfilarial surveys on day blood. Trans. R. Soc. Trop. Med. Hyg. 33, 191–196.

Leong, A.-Y., 1994. Fixation and fixatives. In: Woods, A.E., Ellis, R.C. (Eds.), Laboratory Histopathology, pp. 4.1-1–4.1-26 New York, NY.

Magnis, J., Lorentz, S., Guardone, L., Grimm, F., Magi, M., Naucke, T.J., Deplazes, P., 2013. Morphometric analyses of canine blood microfilariae isolated by the Knott's test enables *Dirofilaria immitis* and *D. repens* species-specific and *Acanthocheilonema* (syn. *Dipetalonema*) genus-specific diagnosis. Parasites Vectors 6, 48.

National Toxicology Program, 2011. NTP 12th Report on Carcinogens. Rep. Carcinog. Carcinog. Profiles 12. iii–499. .

Neer, T.M., Hoskins, J.D., 1989. Clinical experience with ivermectin used as a microfilaricide and for prophylaxis in the dog. In: Otto, G.F. (Ed.), Proceedings of the American Heartworm Symposium' 89. Washington, DC..

Ranjbar-Bahadori, S., Veshgini, A., Shirani, D., Eslami, A., Mohieddin, H., Shemshadi, B., Masooleh, R., 2011. Epidemiological aspects of canine dirofilariasis in the north of Iran. Iran. J. Parasitol. 6, 73–80.

Rhee, J.K., Yang, S.S., Kim, H.C., 1998. Periodicity exhibited by *Dirofilaria immitis* microfilariae identified in dogs of Korea. Korean J. Parasitol. 36, 235–239.

Tilley, L.P., Wilkins, R.J., 1974. The Difil test kit for detection of canine heartworm microfilariae. Vet. Med. Small Anim. Clin. 69, 288–294.

Valenciano, A.C., Cowell, R.L., Rizzi, T.E., Tyler, R.D., 2014. Extracellular organisms. In: Valenciano, A.C., Cowell, R.L., Rizzi, T.E., Tyler, R.D. (Eds.), Atlas of Canine Feline Peripheral Blood Smears. St. Louis, MO, pp. 259–267.

Velasquez, L., Blagburn, B.L., Duncan-Decoq, R., Johnson, E.M., Allen, K.E., Meinkoth, J., Gruntmeir, J., Little, S.E., 2014. Increased prevalence of *Dirofilaria immitis* antigen in canine samples after heat treatment. Vet. Parasitol. 206, 67–70.