



A comparison between different laboratory methods and stains for detection microfilaremic dogs

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Abstract

For microscopical and statistical evaluation of different laboratory methods and stains for detecting circulating microfilariae of *Dirofilaria immitis* in ten naturally infected local stray dog's positive to canine heartworm Ag ELISA kit. All blood samples were examined by three methods modified Knott's technique, serum concentration test, and Türk's solution concentration test and three laboratory stains methylene blue, giemsa, and acridine orange stains were used to dye the precipitate of the modified Knott's technique. Results showed that the slides prepared from the serum were easy to examine (there are no erythrocytes) compared with the slides prepared from the precipitate of the modified Knott's technique. The slides prepared using the Türk's solution were more transparent and freer of cell debris. The length and width of the microfilariae detected by the three methods did not change significantly. Our conclusion from this study is that Türk's solution concentration test and the serum concentration test can be used in place of the modified Knott's technique to detect microfilaremic dogs. Acridine orange staining can also substitute for methylene blue and giemsa stains for faster results.

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Introduction

Dirofilaria immitis infection or canine cardiac dirofilariasis (heartworm disease) is a zoonotic nematode that infects dogs' hearts, pulmonary arteries, and other canids (1). It causes respiratory (pulmonary hypertension, embolization, allergic pneumonitis, eosinophilic granulomas), cardiac (Myocardial hypertrophy, in some animals, congestive heart failure due to high right ventricle afterload), and immune complex glomerulonephropathy (2,3). *D. immitis* is transmitted by various mosquitoes of the genera *Culex*, *Aedes*, *Anopheles* and *Ochlerotatus* (4-6). The laboratory diagnosis of canine cardiac dirofilariasis in live animals is made through the finding of microfilariae (Direct and concentration methods) or direct ELISA or heartworm antigen test kits (7-9) and eventually confirmed by molecular analyses (10,11). The modified Knott's technique is based on the concentration method, and it is a test of choice for the differentiation of species (12). Modified Knott's technique is

performed by diluting 1 ml of blood in 9ml of 2% formalin, which breaks down erythrocytes for better readability and maintains microfilariae for morphometric methods (13). The microfilariae of *D. Units* are currently differentiated by their size in Knott's preparations (7). Previous studies suggest that 2% acetic acid can be used in place of the 2% formalin fixative for a more straightforward disposal of liquid waste and lower potential health risks (14,15). Acetic acid-like formalin has hemolytic properties; it is used as a hemolytic reagent in Türk's solution for the total white blood cell count (16). Acridine orange is an inexpensive fluorescence dye used in laboratory investigation and research applications (17-20). Due to the high sensitivity of the acridine orange stain, it has been widely used for rapid screening of biological samples having external contamination with microorganisms such as bacterial cells in cerebrospinal fluid (21), and malaria in the blood smear (22). The present study aimed to compare modified Knott's technique, serum concentration, and Türk's solution concentration method for

detection of microfilaria of *D. immitis* in dogs and to investigate the suitability of Giemsa and acridine orange stains as a substitute to methylene blue in the modified Knott's technique for discovery of microfilaricidal animals.

Materials and methods

Ethical approve

The study was approved by the institutional animal care and use committee of Veterinary Medicine College, University of Mosul (UM.VET.2021.17 decision number in 6/9/2021).

Sample collection

During the period from October 2021 to January 2022, a five ml of blood was sampled via the cephalic vein from a ten naturally infected local stray dogs (positive with the canine heartworm Ag ELISA kit, DRG International Inc., USA, according to manufacturer's instructions) with an age range of 1 to 7 years. Three milliliters of blood were transferred to a tube containing ethylenediaminetetraacetic acid. The remaining blood was emptied into a non-EDTA (plain tube) and centrifuged at 1500 rpm for 5 minutes to obtain serum.

Modified Knott's technique

Sirois *et al.* (23) described the following modifications, briefly, two milliliters of blood were mixed with 18 milliliters of 2% formalin, then shaken thoroughly to hemolyze the blood and centrifuged at $3000 \times g$ for 5 minutes. The supernatant was discarded, and 3 thin smears were prepared from sediment. The smears were air-dried and fixed in anhydrous (absolute) methanol for 2 minutes, and the first and second smears were stained with methylene blue as mentioned in Coles (13), and Giemsa stains 10% as mentioned in Newton and Wright (24), respectively. The third smear was stained with acridine orange for 2 minutes, as mentioned in Gray *et al.* (25). The first and second smears were examined under a light microscope at $100\times$ and $400\times$ magnifications, but regarding smear was examined under a fluorescent microscope at $100\times$ and $400\times$ magnifications.

Türk's solution concentration test

One milliliter of blood was added to 9 milliliters of Türk's solution (0.2 milliliters of gentian violet 1% + 0.3 milliliters of acetic acid 100% to 0.950 milliliters of distilled water) (13), mixed by inversion and centrifuged at $3000 \times g$ for 5 minutes. The supernatant was discarded. The sediment was observed under a light microscope at $100\times$ and $400\times$ magnifications.

Serum concentration test

A thin smear was prepared, air-dried, fixed with absolute methanol for 2 minutes, then stained with Giemsa stain 10% for 10 minutes, and then examined under a light microscope

at $100\times$ and $400\times$ magnifications Hadi and Al-Zahawi (26). Body length (from the front end to the tail) and diameter with an ocular micrometer of 10 microfilariae in each smear were determined Magnis *et al.* (27), Heidari *et al.* (28).

Statistical analysis

It was performed by two-way analysis of variance to detect the changes in the length and width of microfilariae in the different laboratory tests, and the difference in the percentages between the various stains was assessed by using the Chi-square test using SPSS version 11.5 software for windows. A statistically significant association between variables exists when $P < 0.05$ and at the 95% confidence level.

Results

All concentration tests applied in the current study were positive for 10 samples. Slides prepared using the Türk's solution concentration test were clearer and freer of cellular debris than slides prepared by the modified Knott's technique (Figures 1 and 2). Slides prepared from serum appeared to be easy to examine (without erythrocytes) (Figure 3).



Figure 1: A blood smear reveals *D. immitis* microfilariae in a dog with heartworm disease (modified Knott's technique, $100\times$) under a light microscope.



Figure 2: A blood smear reveals *D. immitis* microfilariae in a dog with heartworm disease (Türk's solution concentration test, $100\times$) under a light microscope.



Figure 3: A blood smear reveals *D. immitis* microfilariae in a dog with heartworm disease (Serum concentration test, 100×) under a light microscope.

There were no significant changes in the length and width of the microfilariae detected by the three methods (Table 1). Examination of stained smears prepared with modified Knott's technique sediment revealed that 100% of the microfilariae were stained with acridine orange (Figure 4), while 70 and 40% of the microfilariae were stained with Giemsa and methylene blue stains, respectively.

Table 1: Measurements of microfilariae (n=10) recorded by various concentration tests

Tests	Mean±SD	
	Length (µm)	Width (µm)
Modified Knott's test	515±90.1*	9.8±2.1*
Türk's solution test	519±88.0*	8.9±1.4*
Serum test	484±92.0*	9.2±1.5*

* No significantly, at P> 0.05.



Figure 4: Acridine orange stain under a fluorescent microscope at 100× magnifications.

Discussion

Accurate diagnosis of *D.immitis* infestation is essential for disease management and control transmission to other animals and humans. The current guidelines from the American Heartworm Society (7) recommend using the

heartworm antigen test and concentration test to detect microfilariae to confirm canine cardiac dirofilariasis.

In the current study, smears prepared by Türk's solution concentration test were clearer and freer of cellular debris than slides prepared using the modified Knott's technique, while the slides prepared from concentrated serum appeared to be easier to examine. In contrast, based on previous studies, the modified Knott's technique remains more valuable than the buffy coat method, wet mount, and thick and thin blood smears in diagnosing canine cardiac dirofilariasis (29,30). In the modified Knott's technique, Ohishiet al. (31) examined acetic acid, acetone, saponin, formalin, benzalkonium hydrochloride, and distilled water for their hemolytic action and established that acetone was most excellent. Recently, acetic acid (15) and glacial acetic acid (32) have become safe and suitable substitutes for formalin.

Türk's solution consists of acetic acid, which is responsible for the hemolysis, and crystal violet to stain the remaining leukocytes, making them easier to see and count (16,33,34). Therefore, Türk's solution concentration test appeared to be an appropriate substitute to modify Knott's technique. Hemolysis is an advantage of the modified Knott's technique due to the hypotonicity of 2% formalin and 2% acetic acid. This improves slide readability by removing most erythrocytes from the sample (13,35). Therefore, the slides prepared from concentrated serum appeared easy to examine (No erythrocytes).

In the modified Knott's technique, acridine orange staining appears to be a favorable alternative to methylene blue. Hansen et al. (36) described acridine orange staining as a fluorochrome stain for the detection of deoxyribonucleic acid (DNA) in bovine babesiosis (37,38). Ravindran et al. (39) described the acridine orange staining technique as an accurate, simple, and rapid screening of many blood samples. It was also reported that time for identification of first parasite was only 1/3 by acridine orange staining as compared to giemsa-stained smears (40,41). This suggests that acridine orange staining is a reliable way to detect cardiac microfilaremia in dogs.

Conclusions

The current study provides uncomplicated, economical, and quick methods (Türk's solution concentration test and the serum concentration test) to detect the microfilaremic dogs, and acridine orange staining is a suitable substitute for methylene blue and Giemsa stains in the modified Knott's technique.

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Conflict of interest

The author declares no conflict of interest in the manuscript.

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مقارنة بين الطرائق المختبرية والصبغات المختلفة للكشف عن المايكروفيلايريا في دم الكلاب

حنين بدران الميلاجي و مآب إبراهيم الفروه جي

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الخلاصة

للتقييم المجهرى والإحصائي للطرائق المختبرية والصبغات المختلفة للكشف عن اليرقات الدقيقة (المايكروفيلايريا) في دم الكلاب المصابة طبيعياً بدودة القلب الدايفيلاريا اميتيس والتي تم تأكيد الإصابة بها باستخدام عدة الاليزا المباشر الخاصة لتشخيص إصابة الكلاب بالدودة القلبية. تم فحص عشرة عينات من الدم بثلاثة طرائق وهي تقانة النوت المحورة واختباري تركيز المصل والتركيز باستخدام محلول الترك. كما استخدمت ثلاث صبغات مختبرية لصبغ راسب تقانة نوت المحورة المثيلين الأزرق، وصبغتي الكيمزا، والأكردين البرتقالي. أظهرت النتائج أن الشرائح المحضرة من المصل سهلة الفحص (لا توجد كريات الدم حمراء)، مقارنة بالشرائح المحضرة من راسب تقانة نوت المحورة. كانت الشرائح التي تم تحضيرها باستخدام محلول الترك أكثر وضوحاً وخالية من حطام الخلايا، كما لا توجد فروقات معنوية بطول وعرض اليرقات التي تم فحصها بالطرائق الثلاثة. استنتجنا من هذه الدراسة أن اختباري تركيز محلول الترك وتركيز المصل يمكن استخدامهما بدلاً عن تقانة نوت المحورة للكشف عن اليرقات الدقيقة في الكلاب المصابة بالدودة القلبية. كما يمكن أيضاً استخدام صبغة الأكردين البرتقالي كبديل لصبغتي المثيلين الأزرق والكيمزا في للحصول على نتائج أسرع.