THE EFFECT OF TEST TYPE, ANTICOAGULANT, STAINS, AND TIME AS FACTORS AFFECTING SIZE MEASUREMENT OF DIROFILARIA IMMITIS

by

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Summary

Microfilaria of Dirofilaria immitis from two heavily infected dogs were detected and measured using the Modified Knott's technique and the Difil Filter test[a]. Variations in the procedures of the two tests were compared as to their effect on the length of the microfilaria. The factors evaluated for their effects were anticoagulant, hemolyzing agent, time elapsed, and stain.

Anticoagulant and time elapsed were found to have no statistically significant effect. Stain was found to have a small, but statistically significant effect. The Difil hemolyzing agent[a] was found to have a highly significant effect on length. It was concluded that microfilaria measured using the Difil test[a] will be reduced to the point where many fall into the reported range for Dipetalonema reconditum. The hemolyzing agent used in the Difil test[a] was found to be the factor causing this size difference.

[a]Difil Test[R] EVSCO Pharmaceutical Corporation, Oceanside, New York 11572.

Introduction

Dirofilaria immitis, the heartworm of dogs is an important problem facing veterinarians. The accurate detection of the microfilaria of *Dirofilaria immitis* is of critical importance in the decision to treat a dog for heartworms. Several criteria such as length, width, shape of the head and tail, and motility are commonly used to differentiate between *Dirofilaria immitis* and *Dipetalonema reconditum*, a nonpathogenic filarid. Length of the microfilaria is one criteria that is easily determined. Two common methods for the detection of the microfilaria of Dirofilaria immitis in the peripheral blood of dogs are the Modified Knott's test and the Difil Filter test[a].

Diagnosis of Dirofilaria immitis is based on the length of the microfilaria measured after running either the Modified Knott's test or the Difil test[a]. The published measurements for Dirofilaria immitis range from 270 to 325 microns[1,2,3,4] and the measurements for *Dipetalonema reconditum* range from 225 to 290 microns[1,2,3,4,5]. These measurements were made using the Modified Knott's test. Measurements made using the Difil Filter test[a] for *Dirofilaria immitis* range from 235 to 267 microns and for *Dipetalonema reconditum* from 150 to 275 microns[5].

It is important to know the effects of time, test

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type, and other variables on the length of the microfilaria of Dirofilaria immitis because the diagnostic tests used for diagnosing heartworms may not be run in a standardized manner. In the offices of veterinarians and in larger laboratories where these tests are run, steps in the procedure are often varied. The first instance where this variation may occur is in the anticoagulant used. There are three commonly used anticoagulants, and no one anticoagulant is always used. Another variable is the time that passes before the tests can be performed on a given blood sample. Many times the blood will have to be refrigerated for a few hours, overnight, or longer. There is also a choice between using the two most commonly used tests; the Modified Knott's test and the Difil Filter Test[a]. The procedures of these two tests differ in the hemolyzing agent and stain utilized. The hemolyzing agent commonly used in the Modified Knott's test is 2% formalin and the hemolyzing agent used in the Difil test[a] is Difil hemolyzing solution[a]. Methylene blue is the stain commonly used in the Modified Knott's test and Difil Stain Solution[a] is used in the Difil test[a]. After all steps in the procedure of each test have been performed the microfilaria are measured. Regardless of which procedure, chemicals, and time period were used the length of the microfilaria are compared to standard values and a diagnosis is made.

A study was conducted by Burt[5] comparing the effects of several factors on the length of microfilaria of Dirofilaria immitis and Dipetalonema reconditum. The two tests, the Modified Knott's and the Difil Filter test[a], were compared using fresh and preserved blood, using Heparin and EDTA as anticoagulants, and using two time periods before the tests were run; immediately and after 18 hours. Burt reports that length measurements made with the Difil Filter test[a] are shorter than those made with the Modified Knott's test. This difference was attributed to the different stain solutions used in the two tests. The other factor that was found by Burt to have an effect was the time elapsed before a given test was run. The microfilaria measured after 18 hours were found to be shorter than those measured immediately.[5]

The objective of this study is to expand the variables used by Burt and determine which factors in each of the t tests would lead to significant changes in the length of the microfilaria.

Materials and Methods

Two dogs from the Texas A and M University College of Veterinary Medicine were used for the study, both dogs were known to have heavy infections of Dirofilaria immitis. Each dog was bled on eight occasions. One of three anticoagulants; EDTA, citrate, or heparin was used. When no anticoagulant was used, 9 mls of 2% formalin or 9 mls of Difil Lysing solution[a] was added. Each sample was divided into three parts; one to be processed immediately, another to be processed in four to five hours, and a third to be processed in twenty four hours. The two samples that were not tested immediately were refrigerated at 4 degrees Celsius. The Modified Knott's test and the Difil test[a] were run on the sample at the end of each time period.

Modified Knott's Test Technique:

The technique for the Modified Knott's test was as follows. Two 10 ml solutions of 1:10 parts of blood and formalin were prepared in centrifuge tubes. After a few minutes the solutions were centrifuged for 10 minutes at 1500 RPM. The supernatant was decanted from both tubes. A drop of 1:1000 methylene blue stain was added to one tube and a drop of the Difil test stain[a] was added to the other tube. After mixing, a drop of the

resulting solution was placed on a slide and covered with a coverslip. These slides were each examined under a microscope. The lengths of 50 microfilaria were measured at 400X magnification with an ocular micrometer.

Difil Filter Test[a] Technique:

The technique for the Difil Filter test[a] involved mixing two solutions of 1:10 parts of blood and Difil lysing solution[a]. Each 10 ml of solution was placed in a 10 cc syringe and forced through the filtering apparatus provided in the kit[a]. Each millipore filter[a] with microfilaria and cellular debris was placed on a slide and stained. One slide was stained with 1:1000 methylene blue and the other with the stain from the filter test kit[a]. These slides were covered with a coverslip and examined under the microscope. The lengths of 50 microfilaria were measured at 400X magnification using an ocular micrometer.

Analysis of Results:

The analysis of the data obtained from performing all of these tests was done using a two way analysis of variance.

Results

Effect of Individual Variables:

The differences in the lengths of the microfilaria between dog 1 and dog 2 were found to be statistically significant. Dog 1 had longer microfilaria on the average. (Table 1). Time was found to have no significant effect on length (Table 1). The three anticoagulants used had no significant effect (Table 1). When the hemolyzing solution was added immediately without the use of an anticoagulant, the difference in the length of the microfilaria was found to be highly significant. Shorter microfilaria were measured on the average when using direct hemolysis versus adding an anticoagulant (Table 1). This effect was the combined effect of the two possible hemolyzing agents, 2% formalin and Difil lysing solution[a]. A significant difference in length was found when the two different test types which used different hemolyzing agents are compared. The Difil test[a] with Difil hemolyzing solution[a] gave shorter microfilaria than did the Modified Knott's test using 2% formalin as a hemolyzing solution (Table 1). The stain had a small, but statistically significant effect, microfilaria stained with Difil test stain[a] were shorter than those stained with methylene blue when the Modified Knott's test was run (Table 1).

Effect of Combinations of Variables:

None of the combinations of variables had a statistically significant effect on length (Table 2).

Discussion

The findings of this study are significantly different from those of a previous study[5]. The finding that time has no effect on the length of the microfilaria is contrary to the results of the study by Burt. Burt reports that microfilaria stored for 18 hours and then measured are smaller than those processed immediately; however, she does not state the numerical value of the size difference or if it was statistically significant. As in Burt's study, the anticoagulant had no effect. When stain is considered the results of this study are in agreement with the results reported by Burt[5]. Stain does have a statistically significant effect on length. Difil stain[a] reduces the size of the microfilaria as compared to methylene blue. The most significant reduction in size of the microfilaria; however, was found to be caused by the hemolyzing agent, not by the stain as Burt concluded[5]. The reduction in size produced by the Difil Stain[a] is small when compared to the effect of the Difil hemolyzing solution[a]. When the combined effects of Difil hemolyzing solution[a] and Difil stain[a] are considered the effect is not significant. Difil hemolyzing solution[a] which is added first to the microfilaria apparently shrinks them to such an extent that the small shrinking effect of the

Difil stain[a] has no additive effect.

Time, anticoagulant and even stain will not modify the length of the microfilaria of Dirofilaria immitis enough to take them below the published range. When the Difil test[a] is used with Difil hemolyzer[a];however, the microfilaria will be reduced in size to the point where many of the microfilaria observed will fall into the published size range of Dipetalonema reconditum.

References

1. Jackson, Ronald F., Gilbert F. Otto : Detection and Differentiation of Microfilaria. Proceedings of the Heartworm Symposium'74. VM Publishing, Inc., Bonner Springs, Kansas : 21-22, 1975.

2. Lindsey, J.R :Diagnosis of Filarial Infections in Dogs. J. Parasit. 48: 321-326, 1962.

3. Newton, W.L., Wright, W.H : The Occurence of a Dog Fillarrid Other Than Dirofilaria immitis in the United States. J. Parasit. 42: 246-258, 1956.

4. Soulsby, E.J.L: Helminths, Arthropods, and Protozoa of Domesticated Animals (Monning). The Williams and Wilkins Company, Baltimore, Md., 1968.

5. Burt, Katherine B: A Comparison of Current Techniques Used in the Diagnosis of Dirofilariasis. The Southwestern Veterinarian, 30: 280-282, 1977.

Table 1

SIGNIFICANT DIFFERENCES TWO WAY ANALYSIS OF VARIANCE

Variable		Means (microns)	
Dog			
	1.	292.9	*
	2.	285.6	
Time			
	0 hours	290.9	
	4 hours	288.2	NS
	24 hours	288.7	
Anticoagulant			
	Citrate	289.9	
	Edta	291.2	NS
	Heparin	292.8	
	Direct Hemolysis (Combination of formalin and Difil lysing solution[a])	283.2	*
Test (Hemolyzer)			
	Knott's test (2% formalin)	313.6	*
	Difil test[a] (Difil hemolyzer[a])	265.0	
Stain			
	Methylene Blue	290.3	*
	Difil Stain[a]	288.2	

* = Statistically significant difference
NS = No statistically significant difference

Table 2

SIGNIFICANT DIFFERENCES OF COMBINED EFFECTS

Variables	Result
Dog X Stain	NS
Dog X Test (hemolyzer)	NS
Time X Anticoagulant	NS
Time X Stain	NS
Time X Test (hemolyzer)	NS
Anticoagulant X Stain	NS
Anticoagulant X Test (hemolyzer)	NS
Stain X Test (hemolyzer)	NS

* = statistically significant difference
NS = no statistically significant difference

Table 3

DOG 1: MODIFIED KNOTT'S TEST USING METHYLENE BLUE STAIN

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	300.7 - 331.7	318.4
	4	294.5 - 334.8	320.6
	24	300.7 - 341.0	319.0
EDTA	0	297.6 - 331.7	317.7
	4	288.3 - 331.7	317.1
	24	310.0 - 331.7	320.0
Heparin	0	297.6 - 344.1	317.2
	4	300.7 - 341.0	320.3
	24	300.7 - 331.7	319.9
None (direct hemolysis)	0	280.7 - 347.2	317.4
	4	303.8 - 344.1	316.2
	24	279.0 - 322.4	306.4

Table 4

DOG 1 : MODIFIED KNOTT'S TEST USING DIFIL STAIN[a]

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	256.0 - 331.7	315.2
	4	300.7 - 347.2	320.5
	24	279.0 - 337.9	318.2
EDTA	0	303.8 - 331.7	316.3
	4	288.3 - 328.6	315.5
	24	297.6 - 328.6	318.9
Heparin	0	297.6 - 341.0	317.3
	4	297.6 - 341.0	318.4
	24	294.5 - 331.7	319.3
None (direct hemolysis)	0	303.8 - 348.6	320.8
	4	300.7 - 337.2	317.2
	24	288.3 - 344.1	311.6

Table 5

DOG 1 : DIFIL TEST[a] USING DIFIL STAIN[a]

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	248.0 - 294.5	269.2
	4	241.8 - 288.3	273.3
	24	254.2 - 294.5	268.5
EDTA	0	251.1 - 285.2	270.1
	4	254.2 - 297.5	272.4
	24	251.1 - 288.3	269.8
Heparin	0	257.3 - 288.3	276.1
	4	248.0 - 285.2	271.1
	24	251.1 - 288.3	269.3
None (direct hemolysis)	0	251.1 - 291.4	267.3
	4	238.7 - 269.7	252.5
	24	238.7 - 266.6	253.5

Table 6

DOG 1 : DIFIL TEST[a] USING METHYLENE BLUE STAIN

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	260.4 - 306.9	279.5
	4	248.0 - 282.1	266.2
	24	260.4 - 331.7	280.3
EDTA	0	232.5 - 285.2	267.2
	4	241.8 - 294.4	265.9
	24	248.0 - 310.0	273,7
Heparin	0	254.2 - 294.5	273.8
	4	260.4 - 288.3	274.7
	24	248.0 - 306.9	269.6
None (direct hemolysis)	0	260.4 - 288.3	1264.7
	4	248.0 - 291.4	259.4
	24	240.0 - 272.8	258.4

Table 7

DOG 2 : MODIFIED KNOTT'S TEST USING METHYLENE BLUE STAIN

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	288.3 - 325.5	312.5
	4	294.5 - 341.0	311.7
	24	282.1 - 341.0	308.3
EDTA	0	297.6 - 325.5	315.0
	4	285.2 - 325.5	310.9
	24	306.9 - 356.5	318.7
Heparin	0	288.3 - 337.9	316.2
	4	288.3 - 325.5	315.9
	24	300.7 - 337.9	319.0
None (direct hemolysis)	0	294.5 - 334.8	304.5
	4	297.6 - 334.8	311.1
	24	300.7 - 334.8	309.8

Table 8

DOG 2 : MODIFIED KNOTT'S TEST USING DIFIL STAIN[a]

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	279.0 - 325.5	305.0
	4	279.0 - 325.5	306.7
	24	294.5 - 325.5	309.2
EDTA	0	291.4 - 328.6	311.3
	4	294.5 - 325.5	299.2
	24	294.5 - 334.8	315.3
Heparin	0	294.5 - 325.5	315.0
	4	279.0 - 322.4	304.5
	24	297.6 - 331.7	309.2
None (direct hemolysis)	0	288.3 - 322.4	309.1
	4	279.0 - 328.6	296.6
	24	291.4 - 322.4	296.4

Table 9

DOG 2 : DIFIL TEST[a] USING DIFIL STAIN[a]

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	248.0 - 279.0	260.6
	4	238.7 - 279.0	258.7
	24	217.0 - 279.0	255.7
EDTA	0	254.2 - 319.3	255.7
	4	248.0 - 285.2	281.0
	24	232.5 - 285.2	265.8
Heparin	0	245.0 - 288.3	260.2
	4	248.0 - 285.2	264.2
	24	214.0 - 282.5	266.2
None (direct hemolysis)	0	248.0 - 310.0	275.8
	4	229.4 - 275.9	242.4
	24	217.0 - 279.0	247.8

Table 10

DOG 2 : DIFIL TEST[a] USING METHYLENE BLUE STAIN

Anticoagulant	Time (hours)	Range (microns)	Mean (microns)
Citrate	0	248.0 - 285.2	263.4
	4	248.0 - 294.5	260.4
	24	232.5 - 279.0	257.6
EDTA	0	248.0 - 322.4	265.1
	4	248.0 - 310.0	260.3
	24	232.5 - 297.6	267.0
Heparin	0	248.0 - 322.4	274.7
	4	248.0 - 297.6	267.8
	24	241.8 - 325.5	270.9
None (direct hemolysis)	0	232.5 - 279.0	257.2
	4	235.2 - 279.0	249.9
	24	229.4 - 279.0	249.7