Histochemical Differentiation of
Dirofilaria immitis and
Dipetalonema reconditum

Laura Chalifoux, B.S., and Ronald D. Hunt, D.V.M.

SUMMARY

Acid phosphatase activity was demonstrated in smears of canine microfilariae by the naphthol AS-TR-phosphate method. Dirofilaria immitis and Dipetalonema reconditum were distinctly and accurately distinguished by this technique. Enzyme activity was restricted to two distinct zones in microfilariae of D. immitis, whereas in Dip. reconditum, acid phosphatase activity was uniformly distributed throughout the organism.

Dirofilaria immitis may cause serious illness in its definitive hosts, whereas Dip. reconditum has not been associated with untoward effects. In both infestations microfilariae are in circulating blood, requiring specific identification to establish diagnosis. Differentiation of circulating microfilariae of the two parasites is difficult. Techniques presently used include observation of activity in fresh drops of blood and differentiation by size and morphologic characteristics in fixed smears or after various methods of concentration and staining. An indirect fluorescent antibody technique has been described and other immunologic methods are being studied.

In studies of filarial infections in monkeys at our laboratory, differences between species of microfilariae were observed in blood smears that had been stained for the demonstration of acid phosphatase activity. This procedure was applied to microfilariae of dogs to assess its value as a means of differentiating microfilariae of D. immitis from Dip. reconditum.

Materials and Methods

Seventy-three dogs, of both sexes and various ages and breeds, of pound origin were examined for filarial parasites.

Approximately 5 ml. of blood was drawn from the cephalic vein of each dog and was allowed to clot. The clot was loosened with an applicator stick and was washed with approximately 5 ml. of distilled water. The water and serum were then poured into a test tube and centrifuged for 5 minutes at about 1,000 g. The supernatant fluid was discarded leaving a drop in the bottom of the tube. A drop of this fluid containing the resuspended sediment was placed on a microscopic slide and examined for microfilariae. Smears were prepared from the sediment of each sample found to contain microfilariae. These were air-dried, fixed in absolute acetone at 4 C. for 1 minute, air-dried and stained for the demonstration of acid phosphatase activity.

From the New England Regional Primate Research Center and Animal Research Center, Harvard Medical School, Southboro, Mass. 01772.

This investigation was supported in part by NIH, USPHS Grant No. FR 00168-09 and No. 5 PO6 RR 00401.

March 1, 1971
The naphthol AS-TR-phosphate method of Barka\textsuperscript{a} was used to demonstrate acid phosphatase activity as follows:

**Reagents**

I. Michaelis veronal acetate buffer, pH 10.0

- Sodium acetate (Na\textsubscript{2}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}·3H\textsubscript{2}O) ............. 9.714 Gm.
- Sodium barbital .......... 14.714 Gm.
- Distilled water .......... 500 ml.

Store at 4 C.

II. Naphthol AS-TR-phosphate

- Naphthol AS-TR-phosphate, sodium salt\textsuperscript{a} .......... 0.05 Gm.
- N\textsubscript{2}N-Dimethyl-
  formamide .......... 5.0 ml.

Prepare fresh

III. Pararosanilin

- Pararosanilin hydro-
  chloride\textsuperscript{b} ............... 1.0 Gm.
- Distilled water .......... 20.0 ml.
- Concentrated HCl .......... 5.0 ml.

Add pararosanilin to water, heat to dissolve. Add HCl and cool. Store at 4 C.

IV. Sodium nitrite, 4%

- Sodium nitrite (NaNO\textsubscript{2}) .......... 4.0 Gm.
- Distilled water .......... 100 ml.

(Keeps well at room temperature or at 4 C.)

V. 1\% methyl green in phosphate buffer

A) 0.2 M sodium phosphate

- Na\textsubscript{2}HPO\textsubscript{4} .......... 28.396 Gm.
- Distilled water .......... 1,000 ml.

B) 0.1 M citric acid

- C\textsubscript{6}H\textsubscript{8}(OH)
  (COOH)\textsubscript{4}·H\textsubscript{2}O .......... 21.011 Gm.
- Distilled water .......... 1,000 ml.

Working solution

- Sol. A .......... 77.1 ml.
- Sol. B .......... 122.9 ml.
- Methyl green .......... 2.0 Gm.

(Keeps well at room temperature)

**Substrate.**—To a Coplin jar add 20 ml. of solution I and 50 ml. of distilled water. To this add 4 ml. of solution II. Mix together in a test tube 3.2 ml. of each of solutions III and IV and add to the mixture in the Coplin jar. Adjust pH to 5.0 with 0.1 N NaOH. The final substrate solution must be prepared fresh before each use. The naphthol AS-TR-phosphate in buffer may be prepared and stored at 4 C. for 4 weeks. Solutions III and IV are stable at 4 C. for more than 6 months.

**Procedure.**—Incubate smears in the substrate for 1 hour at 37 C. or 2 hours at 25 C. Rinse in distilled water. Counterstain in 1\% methyl green (solution V) for 2 to 3 minutes. Rinse in distilled water. Dehydrate first in 95\%, then in absolute ethyl alcohol. Rinse in xylene and mount in permount. The smears are then examined for the precipitated red azo dye indicating acid phosphatase activity.

The procedure may be shortened by eliminating the counterstain, especially if parasites are numerous. The smears may be examined wet for immediate diagnosis.

All samples containing microfilariae were also examined by the method of Newton and Wright,\textsuperscript{b} for the purpose of identification.

Selected blood samples containing microfilariae were stored for varying periods at 4 C., and acetone-fixed smears were stored at −15 C. for various periods to determine the influence of storage on acid phosphatase activity of the microfilariae.

**Results and Discussion**

Seventeen dogs were found to have circulating microfilariae. Two distinct types of microfilariae were observable in smears stained for acid phosphatase activity. In 13 of the smears, the precipitated red azo dye was restricted to the areas of the anal pore and the excretory pore. The areas appeared as 2 bright red bands or spots. In some preparations, less intense staining was observable throughout the body of the microfilariae, but the 2 distinct bands of staining were always clearly visible. Uniform staining of the entire body was not found in any of these microfilariae.

In the remaining 4 smears, the entire body of each microfilaria was stained red.
Fig. 1— *Dirofilaria immitis* with evidence of enzyme activity in areas of excretory pore and anal pore (arrows).

Fig. 2— *Dipelatonema reconditum* with evidence of uniform enzyme activity. Staining is less intense cranial to the excretory pore, but clearly not confined to two distinct bands as seen in microfilariae of *Dirofilaria immitis*. 
In many of these microfilariae, there was less intense staining cranial to the excretory pore, but generally their appearance was uniformly red. Evidence of enzyme activity was never localized into 2 discrete zones.

Identification of the 2 species of microfilariae was based on morphologic and dimensional characteristics. The microfilariae that had 2 distinct areas of acid phosphatase activity (determined to be *D. immitis*) were generally lying in a straight line, had straight tails, and tapering cranial ends. The lengths of these microfilariae ranged from 291.0 µ to 349 µ (mean of 323 µ) and their widths from 5.2 µ to 7.0 µ (mean of 6.2 µ). Microfilariae with enzyme activity throughout the body (determined to be *Dip. reconditum*) tended to lie in a curve, had button-hooked tails, and sides of the head were parallel. The lengths of the microfilariae ranged from 255 µ to 277 µ (mean of 270 µ) and their widths from 4.4 µ to 5.7 µ (mean of 4.9 µ).

It was determined that storage of clotted blood at 4°C for up to 2 weeks did not affect the viability or acid phosphatase activity of the microfilariae. Fixed smears of microfilariae retained acid phosphatase activity when stored at -15°C up to 4 months. Thus the procedure can be performed in stages as convenient.

These results clearly indicate that the difference in acid phosphatase activity between the two species of canine filariids offers an easily observable and reliable method of differentiation. *Dirofilaria immitis* exhibits 2 distinct bright red bands. *Dipetalonema reconditum*, by contrast, appears entirely bright red in the smears. With this method filarial infections may be diagnosed with accuracy by scanning the smears under low power magnification.

References


