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Wilkins et al.

[11] **3,874,851**

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[54] **METHOD AND APPARATUS FOR THE DETECTION OF MICROFILARIAE**

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[52] **U.S. Cl.** ..... **23/230 B,** 23/259, 23/292, 128/2 F, 73/425.6, 195/103.5 R, 195/127

[51] **Int. Cl.** **G01n 33/16,** G01n 1/30

[58] **Field of Search** ..... 23/230 B, 253 R, 259, 292; 195/103.5 R, 127; 128/2 F, 2 G; 424/11; 73/425.6

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[57] **ABSTRACT**

In the detection of microfilariae wherein a blood sample is drawn into a syringe, the blood mixed with an aqueous lysing solution, filtered through a pad, the filter pad stained and examined on a glass slide under a microscope, the improvement which comprises mixing said lysing solution with said blood by drawing said lysing solution into said blood-containing syringe, and discharging the mixture through said filter pad, the blood sample thereby being minimally exposed for contamination. Desirably the lysing solution is employed in about 5 to 15 times the volume of the blood sample and comprises about 1% of a polyoxyalkylene phenol ether, about 1.75% of sodium citratecitric acid wherein the sodium citrate is present in about 3 times the amount of the citric acid and about 0.1% of methyl p-hydroxy-benzoate, the pH being about 6. Advantageously the filter pad comprises a polycarbonate film having pores of about 8 microns in diameter and it is stained on the glass slide with a gentian violet solution containing safranin O and propylene glycol. The apparatus includes a syringe having an open-ended projection which is of such size as snugly to fit in the outlet of the lysing solution dispenser and the inlet of a holder for said filter pad, so as to minimize any possible contamination.

10 Claims, 10 Drawing Figures

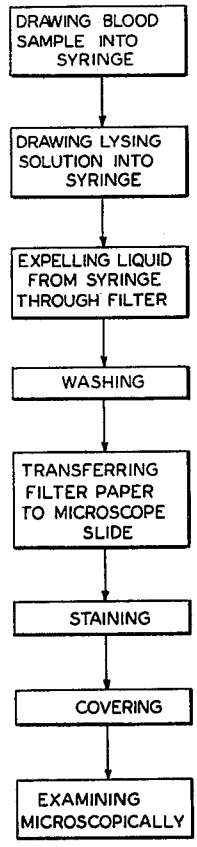


FIG. 1.

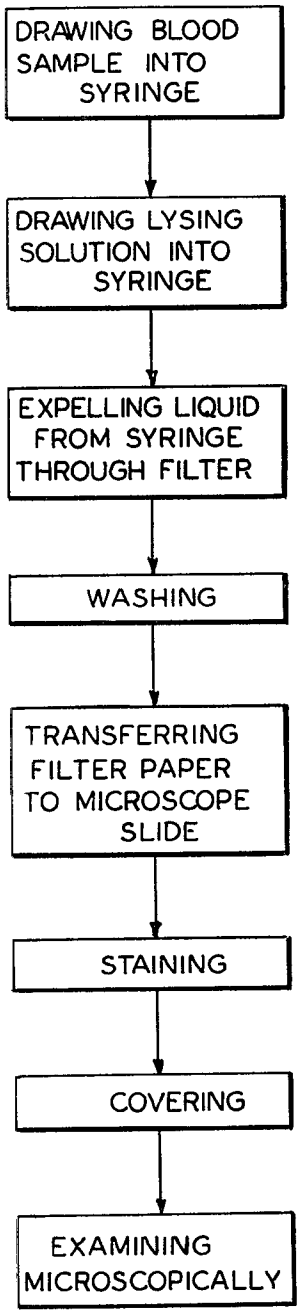


FIG. 2.

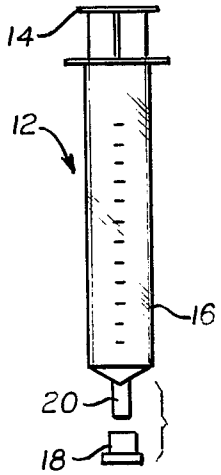


FIG. 3.

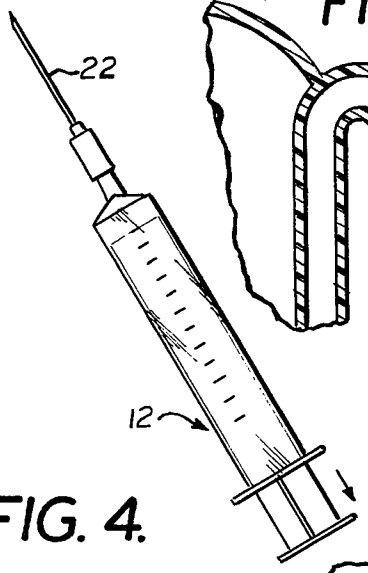


FIG. 5.

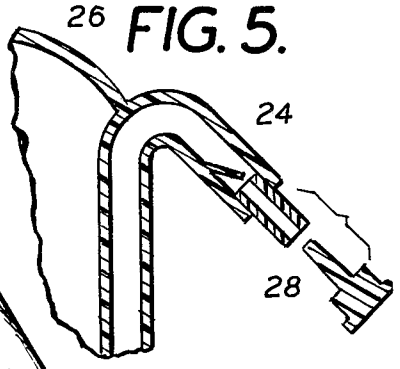


FIG. 4.

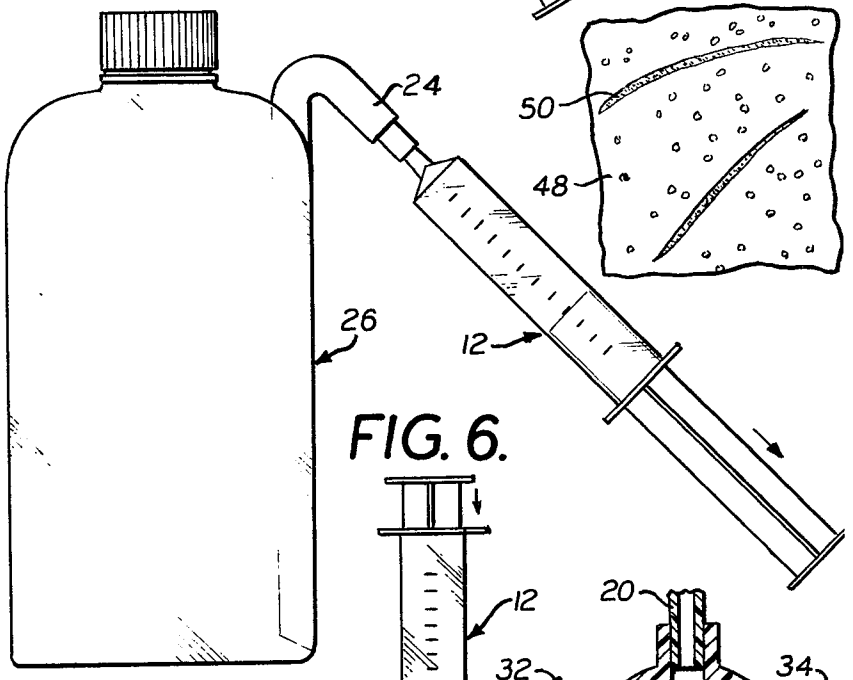
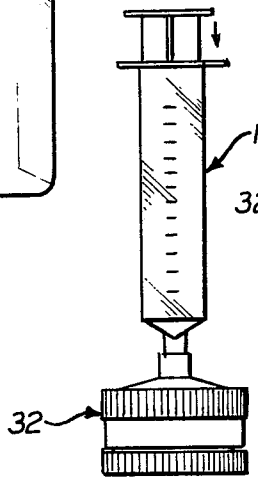


FIG. 10.

FIG. 6.



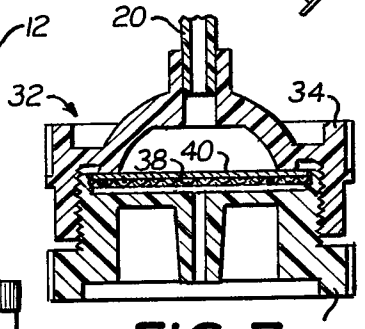


FIG. 7.

FIG. 8.

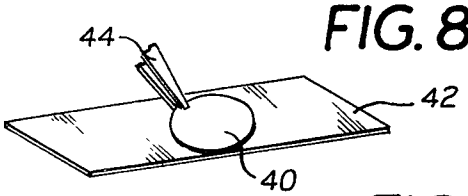
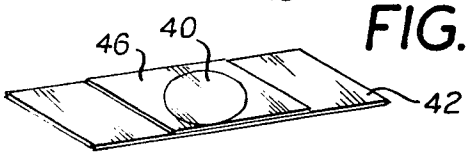


FIG. 9.



METHOD AND APPARATUS FOR THE DETECTION OF MICROFILARIAE

The present invention relates to the detection of microfilariae in blood.

Mammals, particularly small animals such as dogs, have been suffering from heartworm disease, i.e. worms which find their way into the animal's heart with frequently fatal results if not promptly treated. The most satisfactory way of detecting such disease is through examination of a blood sample to detect the presence of *Dirofilaria immitis* microfilariae.

Different procedures have been proposed for such examination. A drop of blood may be placed on a glass slide and directly examined under a microscope. Because the sample is so small, however, microfilariae might not be observed even though present. Use of stains to detect the normally transparent microfilariae helps but other substances present in the blood sample may obscure the microfilariae. In Journal of the American Veterinary Medical Association, Vol. 156, No. 10 (1970), pages 1403 ff., there is described an improved technique wherein a larger blood sample is lysed to solubilize unwanted cellular materials and the mixture is filtered, stained on the filter and examined. This resulted in a marked increase in reliability but the procedure was complicated, required considerable apparatus, and was prone to error due to the possibility of contamination and complication if carried out under less than ideal conditions.

It is accordingly an object of the present invention to provide a simple, reliable process for detecting the presence of microfilariae.

It is a further object of the invention to provide a simple apparatus suited for carrying out such process.

These and other objects and advantages are realized in accordance with the present invention which relates to an improvement in the process of detecting microfilariae wherein a blood sample is drawn into a syringe, the blood mixed with an aqueous lysing solution, filtered through a pad, the filter pad stained and examined on a glass slide under a microscope. Specifically, the improvement comprises mixing said lysing solution into said blood-containing syringe, and discharging the mixture through said filter pad, the blood sample thereby being minimally exposed for contamination.

The invention also provides an apparatus for carrying out the novel process, comprising a syringe having an open-ended projection, an annular needle removably disposed in said syringe for drawing a blood sample therein, a lysing solution dispenser having an outlet for said solution, said outlet being of such size as snugly to receive the open-ended projection of said syringe without said needle, whereby said syringe can be operated to draw said lysing solution into the blood sample within said syringe, a filter holder having an inlet and outlet and means for removably supporting a filter pad therebetween, said inlet being of such size as snugly to receive the open-ended projection of said syringe so as to expel the lysed blood sample onto said filter pad, and a glass slide being of such size as to be received on a microscope viewing stage.

In practicing the invention a blood sample which may be as little as 1 cc or even less is drawn into a syringe through a conventional needle. The sample may be drawn directly from the animal although usually it is drawn from a vial which contains a previously drawn, optionally anti-coagulated, sample of which aliquot portions are to be subjected to a variety of tests.

The syringe needle is removed and the tip of the syringe as a male member is snugly mated with the outlet of a dispenser containing lysing solution, the lysing solution being drawn into the syringe by operating its piston. In this fashion the blood sample does not contaminate the lysing solution within the dispenser which is also kept from normal atmospheric contamination. The syringe barrel thus serves as the only mixing vessel.

Advantageously the dispenser outlet includes a one-way flap- or ball-valve to prevent blood from flowing into the dispenser in the event the syringe piston is moved in the wrong direction.

The lysing solution may be any usually employed but especially satisfactory results are realized when it is an aqueous solution comprising by weight about 0.25 to 2.5% of a non-ionic surfactant, about 1 to 2.5% of an alkali metal citrate-citric acid buffer system wherein the citrate is present in about 2 to 4 times the amount of the citric acid, and about 0.01 to 1% of an antifungal agent, the pH being about 5 to 7. It is employed in about 5 to 15 times the volume of the blood sample, e.g. about 9; smaller amounts may not produce rapid and/or sufficient lysis and larger amounts are unnecessary.

The preferred non-ionic surfactants are polyoxyalkylated phenol ethers such as polyoxyethylene octylated phenol ether. Suitable materials are sold by Rohm and Haas under the tradenames Triton X-100 and Triton X-405. It solubilizes the blood cell components more quickly and reliably than other surface active agents. Preferably it is employed in about 1% by weight of the solution although, as noted, it can be employed in about 0.25 to 2.5%. Lower amounts produce insufficient or too slow lysis; larger amounts result in foaming which interferes with the subsequent filtration.

The citrate may be potassium or other soluble salt, although preferably it is sodium citrate. The citrate-citric acid system serves to maintain the pH at the desired level of about 5 to 7, advantageously about 5.5 to 6.5 and preferably about **6.** More acid systems may result in precipitation of the iron of the hemoglobin which would interfere with the microscopic observation. Less acid systems result in more viscous solutions with much cellular debris which will effect less selective filtration. The citrate-citric acid also serves the beneficial function of an anticoagulant.

The overall amount of citrate-citric acid may range from about 1 to 2.5% although preferably it is more than about 1 and especially about 1.75%. Lesser amounts result in too little anticoagulation and too low a pH. Higher amounts are wasteful and may exceed the solubility of both components. The citrate is advantgeously present in about 2 to 4 times, preferably about 3 times, the weight of the citric acid for the desired effect.

Coloring agents, stabilizers, and the like may also be present. A particularly suitable component is an antifungal agent to prevent contamination of the lysing solution itself. Particularly suitable for this purpose is methyl p-hydroxybenzoate (methylparaben) employed in about 0.01 to 1%, advantageously about 0.05 to 0.2% and preferably about 0.1% by weight. The balance of the solution is preferably deionized or distilled water.

After allowing the blood sample and lysing solution to mix for a few seconds in the barrel of the syringe, the tip of the syringe is inserted in mating relationship into the inlet of a filter holder having an inlet and outlet and means for removably supporting a filter pad therebetween. The syringe plunger is actuated to force the lysed blood sample through the filter pad, retaining thereon insoluble material which will include the microfilariae, if present. The syringe may be used to force wash water once or twice through the filter pad to ensure removal of any debris.

The filter pad is preferably a membrane or film of carefully controlled pore size, e.g. about 2.5 to 10 microns and advantageously about 5 to 8 microns. Smaller pores result in slow filtration and may remove some of the cellular debris which is undesirable. Too large a pore size will obviously pass the microfilariae. A General Electric Nucleopore polycarbonate film having pores of about 8 microns is especially suitable.

After filtration the membrane is removed and placed on a glass microscope slide. Conventional stains may be used to facilitate detection of the normally transparent microfilariae but it has been found that methylene blue is far less satisfactory than gentian violet which gives sharper and deeper coloration. The gentian violet is preferably employed in combination with safranin 0 and a non-volatile humectant such as a polyhydroxyalkane, e.g. a lower alkylene glycol or polyglycol, glycerine or the like. Propylene glycol is particularly satisfactory. This serves to prevent drying out of the filter pad when placed between two glass plates and viewed under the microscope. The two glass plates may be squeezed to expel any excess liquid.

By staining the filter pad or the glass slide rather than in the filter holder, less stain is used and the excess stain remains on the pad rather than being washed therethrough. In addition there is less mess and no coloration of the filter holder.

Viewing is thereafter conducted in conventional fashion. It is obvious that detection of microfilariae will be simplified because of concentration from the larger volume of blood and the freedom from contamination.

The invention will now be described with reference to the accompanying drawing wherein:

FIG. **1** is a flowsheet of the steps involved in the process in accordance with the present invention;

FIG. **2** is a side view of the syringe at the outset of the process;

FIG. **3** is a view of the syringe and hypodermic needle after drawing a blood sample;

FIG. **4** is a view of the syringe coupled to a lysing solution dispenser;

FIG. **5** is an enlarged sectional view of the outlet of the dispenser of FIG. **4**;

FIG. **6** is a view of the syringe discharging its contents through a filter holder;

FIG. **7** is a longitudinal sectional view through a portion of FIG. **6**;

FIG. **8** is a perspective view of a glass slide carrying a filter pad during the act of staining;

FIG. **9** is a view of the slide of FIG. **8** surmounted by a glass plate; and

FIG. **10** is an enlarged view of a portion of what is seen when viewing the slide of FIG. **9** on a microscope.

Referring now more particularly to the drawings, in FIG. **2** there is shown a syringe **12** comprising a piston or plunger **14** fitting within a barrel **16.** A cap **18** is removably fitted over the tip or projection **20** of the syringe **12.** The cap **18** is removed and replaced by a hypodermic needle **22** (FIG. **3**) which is inserted into a vial (not shown) containing a blood sample or into an animal. The plunger **14** is retracted, drawing a blood sample into the barrel **16.**

The needle **22** is then removed and the tip **20** is mated with the outlet **24** of a plastic wash bottle **26** (FIG. **4**). As seen in FIG. **5** the outlet **24** in inoperative position has a cap **28** which is removed when the syringe is to be attached. A one-way valve, e.g. a flap **30** prevents blood from the syringe **12** being expelled into the dispenser **26.** Instead, the syringe plunger **14** is further retracted to draw lysing solution into the barrel **16** to mix with the blood sample.

The syringe projection **20** is removed from the dispenser outlet **24,** the cap **28** replaced on the dispenser, and the projection **20** is mated with the inlet of a filter assembly **32** as shown in FIG. **6.** As shown in FIG. **7,** the assembly **32** comprises threadedly engaging inlet and outlet members **34** and **36,** respectively. A wire screen support **38** is mounted in one of the members, e.g. outlet **36** and it supports a filter membrane **40.** The syringe contents are expelled through the membrane **40,** any solids therein being retained on the membrane. Desirably the syringe is uncoupled, water drawn therein and expelled into the assembly **32** as a wash.

The membrane **40** is placed on a glass slide **42** (FIG. **8**) and a stain is applied through a dropper **44,** whereafter a second glass plate **46** is superposed (FIG. **9**) and the prepared slide is viewed under a microscope (not shown). As seen in FIG. **10** there can be seen holes **48** of fairly regular size, which are the openings in the membrane **40.** There are also visible stained elongated microfilariae **50.**

The invention will now be described in the following illustrative example which was carried out with the apparatus of the drawing.

EXAMPLE

1 cc of blood was drawn from a stoppered vial containing blood drawn from a heartworm-infected dog, using a 25 cc syringe equipped with a standard needle. The needle was removed and the syringe tip inserted into the uncovered outlet of a lysing solution dispenser containing lysing solution at a pH of **6** and of the following composition;

octyl phenoxy polyethoxy ethanol (Triton X-100)	10	cc
sodium citrate	13.2	grams
citric acid	4.4	grams
methyl p-hydroxy-benzoate	1	gram
Yellow No. 5 (FDSC) coloring agent	1	gram
deionized water to	1	liter

9cc of lysing solution were withdrawn and after 5 seconds the syringe tip was placed in the inlet of a Gelman membrane filter holder whose screen had been provided with a General Electric Nucleopore polycarbonate filter having an **8** micron pore size. The lysed solution was expelled through the filter membrane and the syringe was used to effect two water washes of 10 cc each time. The filter membrane was removed, placed on a glass slide and a few drops of staining solution were applied, the staining solution having been formulated as set out hereinbelow. A second glass slide was placed over the filter membrane, the glass slides

squeezed together and expelled liquid wiped away. The slides were then placed under a microscope and microfilariae were readily visible.

Preparation of Staining Solution

A.	gentian violet	3.0	grams
	ammonium oxalate	0.8	gram
	ethyl alcohol, 95%	20	cc
	distilled water to	100	cc
B.	safranin O (Certified Biological Stain C.I. 50240)	0.25	gram
	ethyl alcohol, 95%	10	cc
	distilled water to	100	cc

5 parts by volume of A were mixed with 95 parts of B, the mixture in turn mixed with one-half its volume of propylene glycol and filtered.

It will be appreciated that the instant specification and example are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

What is claimed is:

1. In the detection of microfilariae wherein a blood sample is drawn into a syringe, the blood mixed with an aqueous lysing solution, filtered through a pad, the filter pad stained and examined on a glass slide under a microscope, the improvement which comprises mixing said lysing solution with said blood by drawing said lysing solution into said blood-containing syringe, and discharging the mixture through said filter pad, the blood sample thereby being minimally exposed for contamination.

2. The process of claim **1,** wherein said aqueous lysing solution comprises by weight about 0.25 to 2.5% of a non-ionic surfactant, about 1 to 2.5% of an alkali metal citrate-citric acid buffer system wherein the citrate is present in about 2 to 4 times the amount of the citric acid, and about 0.01 to 1% of an antifungal agent, the pH being about 5 to 7.

3. The process of claim **2,** wherein said aqueous lysing solution is employed in about 5 to 15 times the volume of the blood sample and comprises about 1% of a polyoxyalkylene phenol ether, about 1.75% of sodium citrate-citric acid wherein the sodium citrate is present in about 3 times the amount of the citric acid, and about 0.1% of methyl p-hydroxy-benzoate, the pH being about 6.

4. The process of claim **1,** wherein said filter pad comprises an inert permeable membrane having pores of about 2.5 to 10 microns in diameter.

5. The process of claim **4,** wherein said filter pad comprises a polycarbonate film having pores of about 8 microns in diameter.

6. The process of claim **1,** wherein said filter pad is stained on said glass slide with a gentian violet solution.

7. The process of claim **6,** wherein said gentian violet solution further contains safranin 0 and a nonvolatile humectant.

8. The process of claim **3,** wherein said filter pad comprises a polycarbonate film having pores of about **8** microns in diameter, and said film is stained on said glass slide with a gentian violet solution containing safranin 0 and propylene glycol.

9. An apparatus for carrying out the detection of microfilariae in a blood sample comprising a syringe having an open-ended projection, an annular needle removably disposed in said syringe for drawing a blood sample therein, a lysing solution dispenser having an outlet for said solution, said outlet being of such size as snugly to receive the open-ended projection of said syringe without said needle, whereby said syringe can be operated to draw said lysing solution into the blood sample within said syringe, a filter holder having an inlet and outlet and means for removably supporting a filter pad therebetween, said inlet being of such size as snugly to receive the open-ended projection of said syringe so as to expel the lysed blood sample onto said filter pad, and a glass slide for receiving said filter pad, said glass slide being of such size as to be received on a microscope viewing stage.

10. The apparatus according to claim **9** including a one-way valve in said outlet of said lysing solution dispenser to prevent accidental expulsion of blood from said syringe into said dispenser.

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