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Title: Transmission of "Salmon Poisoning" Disease to Dogs by

Nanophyetus salmincola Eggs

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The presence of the etiologic agent of "salmon poisoning" disease, Neorickettsia helminthoeca, was demonstrated in eggs of the trematode vector, Nanophyetus salmincola. Three dogs were given 100,000 and one dog 82,000 ground fluke eggs by intraperitoneal injection. The four animals developed "salmon poisoning" disease and died. One of these dogs had been given 100,000 intact eggs previously; this inoculum did not produce the disease. Two dogs that received either intact or ground adult flukes intraperitoneally also died from "salmon poisoning" disease. Noninjected control dogs remained healthy in all instances. Lymph nodes removed from a dog that had been injected with 82,000 ground eggs and had died from the disease were injected intraperitoneally into a susceptible dog. After this dog developed signs of "salmon poisoning" disease, it was given antibiotic therapy and allowed to

recover. This animal was shown to be immune to the disease by challenge with N. salmincola metacercariae from fish. A susceptible control dog given the same number of metacercariae developed the disease and died. Intracytoplasmic inclusion bodies were seen in stained lymph node cells from all dogs that developed the disease.

Transmission of "Salmon Poisoning" Disease
to Dogs by <u>Nanophyetus</u> <u>salmincola</u> Eggs

by

Peter Albert Nyberg

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TRANSMISSION OF "SALMON POISONING" DISEASE TO DOGS BY <u>NANOPHYETUS SALMINCOLA</u> EGGS[1]

INTRODUCTION AND LITERATURE REVIEW

"Salmon poisoning" disease (SPD) has been produced in susceptible dogs by intraperitoneal (IP) injection of all stages of the trematode vector, <u>Nanophyetus salmincola</u>, except the eggs and miracidia. Simms et al. (1931) and Simms and Muth (1933) produced SPD in dogs by injecting intact and ground, respectively, adult flukes that were recovered from intestines of infected dogs. Simms et al. (1932) produced the disease in dogs by injecting metacercariae from salmonid fishes, and Philip et al. (1954b) by injecting ground livers of the snail host <u>Oxytrema silicula</u> that contained cercariae. Transovarian transmission of the etiologic agent, <u>Neorickettsia helminth-oeca</u>, into fluke eggs was suggested by Philip et al. (1954a) and Philip (1958). These authors indicated that even though experimental proof was lacking, the circumstantial evidence was overwhelmingly in favor of this type of transmission in the fluke. Philip et al. (1954b) tested for presence of the agent in fluke eggs by injecting (IP) 14,000 ground eggs into one dog and 51,000 into another. Neither

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[1] A portion of this thesis has been previously published according to the following reference.

Nyberg, P. A., S. E. Knapp and R. E. Millemann. 1967. "Salmon Poisoning" Disease. IV. Transmission of the disease to dogs by <u>Nanophyetus salmincola</u> eggs. J. Parasit. 53:694-699.

dog developed the disease. Philip (1958) suggested that this experiment should be repeated using larger numbers of eggs and different methods of preparation; however, no additional attempts have been made. Thus, there has continued to the present time an uncertainty as to where N. helminthoeca entered the fluke's life cycle.

This thesis reports the results of a study undertaken to determine if the causative agent of SPD is present within the eggs of N. salmincola.

MATERIALS

Experimental Animals

Thirteen dogs were used in these experiments. They were obtained when four to five weeks old from the Benton County Oregon pound. Procedures for handling dogs in the kennel, and for recording data, were similar to those reported by Andersen (1964) and McKelvie and Schultz (1964). Body temperature and weight of each dog were recorded daily at approximately 9:00 a.m. All of the animals used in Experiments I and II were female littermates of pointer-beagle origin with exception of donor dog (D-I) for Experiment I; this animal was also a female and of approximately the same age as the others but a mongrel. Dogs used in Experiment III were all female littermates of labrador-pointer origin with exception of (D-8) and (D-10). Dog (D-8) was a male littermate of other dogs used in Experiment III, and (D-10) was a mongrel male of approximately the same age as the others.

The animals were not vaccinated against any canine diseases and had not been exposed to SPD prior to these experiments. All animals were at least four months old when used in an experiment.

Metacercariae

Metacercariae were recovered from the kidneys of naturally

infected rainbow trout (<u>Salmo gairdneri</u>) and coho salmon (<u>Oncorhyn-</u>
<u>chus kisutch</u>) according to procedures used by Gebhardt et al.
(1966). They were used to infect donor animals (D-I, D-II, and
D-III) for the three experiments, and for challenge of a nonexposed
control animal (D-10) and of a dog (D-8) that had been injected intra-
peritoneally with a suspension of infected dog lymph nodes. Meta-
cercariae were given to dogs by stomach tube.

<div align="center">

Adult Flukes and Fluke Eggs

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Adult flukes and fluke eggs were recovered from donor ani-
mals by repeated washing and sedimentation of intestinal contents in
distilled water at 20 C. The procedure used was the one reported
by Gebhardt et al. (1966) for recovery of metacercariae from fish,
but modified to include the use of additional Tyler screen scale
sieves in a series of 42, 65, 100, 150, 200, and 270 meshes to the
inch. Flukes were recovered from the 65 and 100 mesh screens,
and eggs from the 270 mesh screen. Flukes and eggs, immediately
after recovery, were suspended in Hank's balanced salt solution
(BSS) (Merchant et al., 1964) and kept in an ice bath until they were
injected. The time required for recovery of flukes and eggs (until
suspended in Hank's BSS) was approximately four hours.

Flukes and eggs were washed several times in sterilized
Hank's BSS. The container of flukes and eggs was placed in an ice

bath after each washing. Intact flukes and eggs used in Experiment I were taken directly from the sample in the ice bath and injected into dogs immediately after washing in Hank's BSS was completed. Other suspensions of flukes and eggs, after several washings in Hank's BSS, were ground in separate glass (Ten Broeck) tissue homogenizers immersed in an ice bath. The ground material was immediately injected into dogs.

Lymph Nodes

Mesenteric lymph nodes were removed aseptically when dogs were necropsied. A small part of this tissue was used to prepare impression smears that were stained according to the technique reported by Giménez (1964) for rickettsiae in yolk-sac cultures (see Appendix). The remaining tissue was frozen and stored at -70 C.

In Experiment III lymph node tissue from an infected dog (D-9) was placed in a glass tissue grinder containing 3 ml of Hank's BSS. The tissue was ground at a temperature of approximately 5 C for 5 minutes and then immediately injected IP into a susceptible dog (D-8).

EXPERIMENTAL PROCEDURES AND RESULTS

The basic design and some results of the three experiments are shown in Figure 1. Complete procedures and results are given under the corresponding experiment numbers. Body temperature and weight curves for the individual dogs are shown in Figures 2-15.

Experiment I

Procedure (Figure 1)

Donor dog (D-I) was given 22,000 $\underline{N}$. salmincola metacercariae and killed 18 days later. Approximately ten thousand adult flukes and 200,000 fluke eggs were recovered from the intestinal contents and prepared for injection. Flukes and eggs were each divided into two equal lots. Flukes and eggs of one lot were ground, and those in the other lot were left intact.

Four dogs (D-1, D-2, D-3, and D-4) were injected IP with 5,000 intact flukes, 5,000 ground flukes, 100,000 intact eggs, and 100,000 ground eggs, respectively. A fifth dog (D-5) served as a noninjected control.

Results

"Salmon poisoning" disease (SPD) developed in four of the six dogs used in this experiment, namely, in the donor dog (D-I), the

SALMONID FISH

METACERCARIAE
N. salmincola

EXPERIMENT
NUMBER

DONOR DOGS

D-I   D-II   D-III

MATERIALS

flukes   fluke eggs   fluke eggs   fluke eggs

5,000 intact   5,000 ground   100,000 intact   100,000 ground   Control   100,000 ground   Control   Control   82,000 ground   Control

RECIPIENT DOGS

D-1   D-2   D-3   D-4   D-5   D-6   D-7   D-8   D-9   D-10

RESULT

SPD   SPD   NO SPD   SPD   NO SPD   SPD   NO SPD   NO SPD   SPD   NO SPD

CHALLENGE

100,000 ground   100,000 ground   4,000 stomach tubed   lymph node

RESULT OF CHALLENGE

SPD   SPD

CHALLENGE II

RESULT

IMM   SPD

SPD = "Salmon Poisoning" Disease;   IMM = Immune;   = Killed when moribund;

= Died of SPD;   = Recovered from SPD after antibiotic therapy;   = No signs of disease;

FIGURE 1.   Transmission of "salmon poisoning" disease to dogs by eggs of *Nanophyetus salmincola*. Schema of Experiments I, II, and III.

Figures 2-15.   Figures 2-7, 8-11, 12-15, represent Experiments
I, II, and III, respectively.   Comparative body
temperatures and weights between principal and
control dogs.   2.   Donor dog (D-I) for Experiment I,
given 22,000 metacercariae by stomach tube.   3-6.
Injected IP with 5,000 intact flukes (D-1), 5,000
ground flukes (D-2), 100,000 intact fluke eggs (D-3),
and 100,000 ground fluke eggs (D-4), respectively.
7.   Noninjected control (D-5) for Experiment I.
8.   Donor dog (D-II) for Experiment II, given 50,000
metacercariae by stomach tube.   9-10.   Injected IP
with 100,000 ground fluke eggs each (D-3, D-6).
11.   Noninjected control (D-7) for Experiment II.
12.   Donor dog (D-III) for Experiment III, given
15,000 metacercariae by stomach tube.   13.   Dog
(D-9) injected IP with 82,000 ground fluke eggs.
14.   Dog (D-8) injected IP with infected lymph nodes,
received antibiotic therapy, and challenged with
4,000 metacercariae.   (Daily body weights and
temperatures were taken to day 78.)   15.   Control
dog (D-10) for Experiment III, given 4,000 meta-
cercariae by stomach tube.
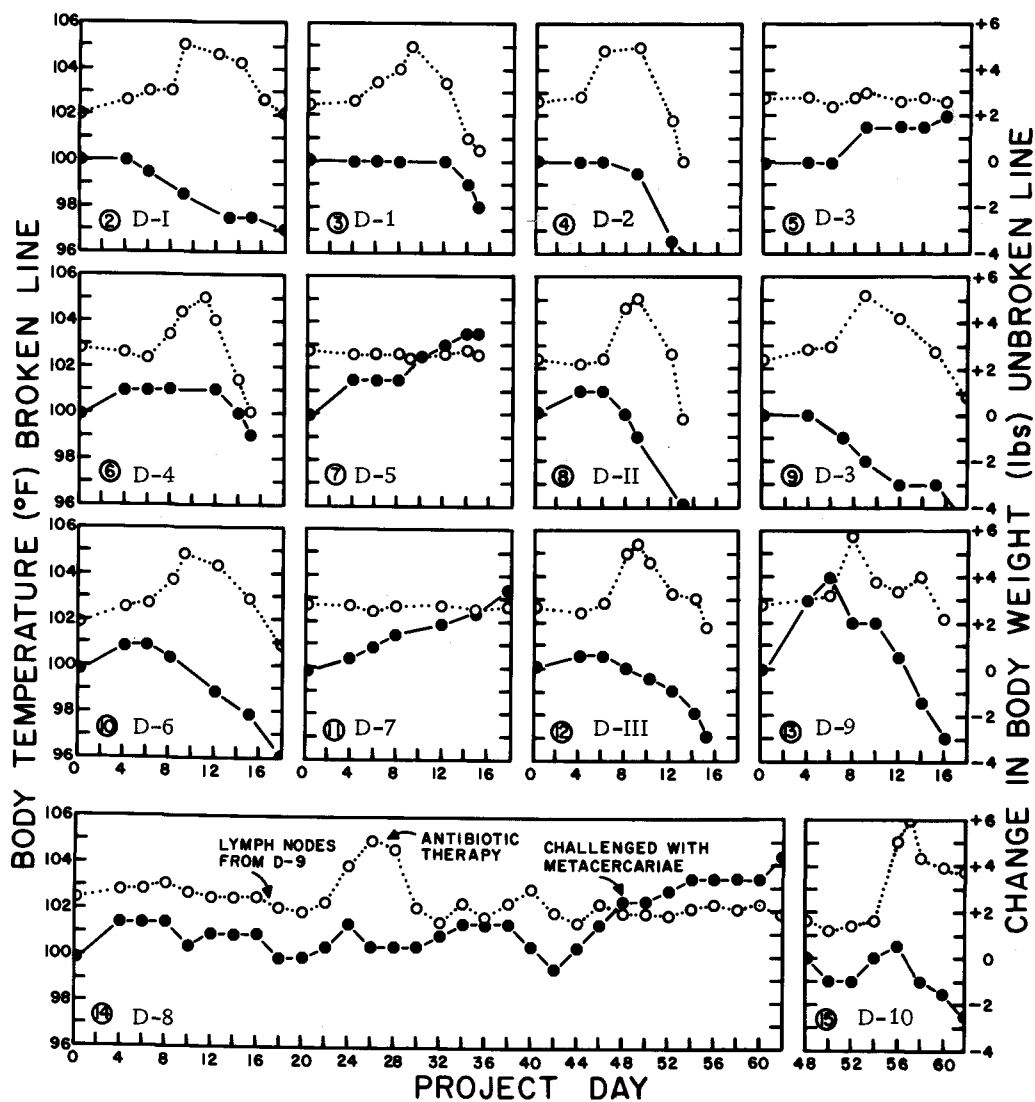
dogs injected with intact or ground adult flukes (D-1, D-2) and in dog (D-4) that received ground fluke eggs (Figure 1). Dog (D-I) was killed 18 days after infection and dogs (D-1, D-2, D-4) died 15, 13 and 15 days after infection, respectively. Daily body weight and temperature data of the principals (D-I, D-1, D-2, D-4) are shown in Figures 2, 3, 4 and 6, respectively. The dog (D-3), that received intact fluke eggs, and the control dog (D-5) did not develop signs of disease (Figure 1). Body temperature and weight changes for these two dogs are given in Figures 5 and 7.

Experiment II

Procedure (Figure 1)

Donor dog (D-II) was given 50,000 N. salmincola metacercariae. The animal developed SPD, was killed 13 days later and fluke eggs were recovered from its intestinal contents. The eggs were divided into two lots of approximately 100,000 each and kept in a refrigerator until used. Forty-eight hours after being collected the eggs were injected (IP) into dogs (D-3) and (D-6). Dog (D-3) had survived injection with intact eggs 28 days earlier in Experiment I. A noninjected dog (D-7) was the control.

Results

The dogs given ground fluke eggs (D-3, D-6) developed signs of SPD and died 18 days after injection (Figures 1, 9, 10). The control (D-7) did not develop signs of disease (Figure 11).

Body temperature and weight changes for animals used in this experiment are shown in Figures 8-11.

Experiment III

Procedure and Results (Figure 1)

The purpose of this experiment was to confirm that the infectious agent in Nanophyetus salmincola eggs was Neorickettsia helminthoeca.

Fifteen days after the donor dog (D-III) was given 15,000 N. salmincola metacercariae, it was killed and fluke eggs were recovered from the intestinal contents. Approximately 82,000 eggs were recovered, cleaned, ground, and injected (IP) into dog (D-9). A susceptible dog (D-8) was used as a noninjected control. Signs of SPD were observed in dog (D-9) and it died 16 days after infection (Figures 1, 13). No signs of disease were seen in the control for days 0-16 (Figures 1, 14). Mesenteric lymph nodes were removed from dog (D-9) and a suspension of them were injected (IP) into dog (D-8) on day 17 (Figures 1, 14). Seven days after dog (D-8) was

injected with this suspension of lymph nodes, a rise in body temperature and signs of SPD were observed (Figure 14). Terramycin was given to this dog for day 27 through 30 at a dosage of 25 mg/lb body weight. Signs of disease disappeared on day 32 and 16 days later (day 48) it was given 4,000 metacercariae. A susceptible control dog (D-10) was also given 4,000 parasites. Signs of disease were not observed in dog (D-8) after challenge with metacercariae (Figures 1, 14). "Salmon poisoning" disease developed in the control dog (D-10) and the animal died 14 days (day 62) after it was given metacercariae (Figures 1, 15). Fluke eggs appeared in the feces four days after dogs (D-8) and (D-10) were given metacercariae. Lesions of SPD, as described by Cordy and Gorham (1950), were found in all infected dogs at necropsy.

Hadlow (1957) reported lesions in the central nervous system of dogs with SPD. Animals used in these experiments, however, were not examined for such lesions. Intracytoplasmic inclusion bodies similar to those described by Cordy and Gorham (1950) and Philip (1955) were found in lymph node impression smears from each infected dog (see Appendix for identification techniques).

DISCUSSION

This is the first report of "salmon poisoning" disease in canines produced by injection of N. salmincola eggs. Thus, the hypothesis first proposed by Philip et al. (1954a), that N. helminthoeca is carried transovarially in the fluke vector, is confirmed. Also, it is clear that the agent in the egg, as well as in all other fluke stages, is in an infectious state, as first postulated by Philip (1962), and not in an occult state. This is in contrast with helminth-borne swine influenza. Virus of the latter disease has not been detected in lungworm eggs (Philip, 1958).

Results of this study suggest that the SPD disease agent is located within the egg rather than on the exterior surface of the shell, because the disease was produced by ground but not by intact eggs. Additional evidence for intra-egg transmission of the agent was provided by Philip (1955), who showed that the agent was present in fluke stages within the snail host; its presence there would be difficult to explain if the agent were merely a contaminant on the eggs' exterior surfaces.

Findings reported here do not agree with those of Philip et al. (1954b), who were unable to produce the disease in two dogs which received 14,000 and 51,000 ground eggs IP. They prepared their inocula either by "carefully separating" the eggs from the flukes,

or by allowing flukes to discharge eggs during a period of 48 hours at 37 C. Their inoculum consisting of 14,000 eggs was stored in tap water at room temperature for one month before it was injected into the dog. The 51,000 eggs that constituted their other inoculum were ground and injected 48 hours after they were recovered. Our inocula were prepared from eggs recovered from intestinal contents of dogs with SPD, and injections were made either on the same day that eggs were collected (Experiments I and III), or 48 hours after collection (Experiment II). The eggs for our studies were kept in an ice bath at 5 C from the time they were recovered until they were injected. In all of our experiments, eggs were injected within five minutes after they were ground. We used glass (Ten Broeck) tissue homogenizers to grind the eggs which were suspended in Hank's BSS. Philip et al. (1954b) used agate mortars and pestles to grind the eggs which were suspended in either heart broth infusion (14,000 inoculum) or beef albumin in buffered saline (51,000 inoculum).

One or a combination of the differences in methods used in the two studies could adequately explain the dissimilar results obtained. This is highly probable in view of the unstable nature of the agent as reported by Philip et al. (1954b), Philip (1955) and as observed by the author in previous experiments (unpublished). It was found that adult flukes suspended overnight in buffered saline or distilled water,

and either ground prior to injection or left intact, failed to produce
SPD. Inconsistent results were also obtained with lymph nodes
from infected dogs that were treated in a similar manner. Thus,
the stability of the agent may be affected by the nature of the sus-
pending medium or by the length of time that the material is left in
the medium.

Failure to produce the disease in dogs by injection of intact
eggs suggests that the infectious agent must be released by egg
disintegration. Either this does not occur in vivo or if it does the
agent may have already been destroyed by toxic products of the dis-
integrating egg contents. Under both conditions development of the
disease would be prevented. The fate of intraperitoneally injected
intact fluke eggs merits investigation.

BIBLIOGRAPHY

Andersen, A. C. 1964. Air conditioned cages designed to minimize kennel problems. Laboratory Animal Care 14:292-303.

Cherry, W. B., M. Goldman and T. R. Carski. 1960. Fluorescent antibody techniques. Washington, D. C., United States Government Printing Office. 73 p. (U. S. Public Health Service. Publication No. 729)

Clark, H. F. and C. C. Shepard. 1963. A dialysis technique for preparing fluorescent antibody. Virology 20:642-644.

Cordy, D. R. and J. R. Gorham. 1950. The pathology and etiology of salmon disease in the dog and fox. American Journal of Pathology 26:617-637.

Gebhardt, G. A., R. E. Millemann, S. E. Knapp and P. A. Nyberg. 1966. "Salmon poisoning" disease. II. Second intermediate host susceptibility studies. Journal of Parasitology 52:54-59.

Giménez, D. F. 1964. Staining rickettsiae in yolk-sac cultures. Stain Technology 39:135-140.

Hadlow, W. J. 1957. Neuropathology of experimental salmon poisoning of dogs. American Journal of Veterinary Research 18:898-908.

Levy, H. B. and H. A. Sober. 1960. A simple chromatographic method for preparation of gamma globulin. Proceedings of the Society of Experimental Biology and Medicine 103:250-252.

Lillie, R. D. 1965. Histopathologic technique and practical histochemistry. New York, McGraw-Hill Company. p. 584.

Livingston, C. W., Jr. and R. W. Moore. 1962. Cytochemical changes of blue tongue virus in tissue culture. American Journal of Veterinary Research 23:701-713.

Merchant, D. J., R. H. Kahn and W. H. Murphy, Jr. 1964. Handbook of cell and organ culture. Minneapolis, Burgess Publishing Company. 263 p.

McKelvie, D. H. and F. T. Schultz. 1964. Methods of observing and recording data in long-term studies on beagles. Laboratory Animal Care 14:118-124.

Nyberg, P. A., S. E. Knapp and R. E. Millemann. 1967. "Salmon poisoning" disease. IV. Transmission of the disease to dogs by Nanophyetus salmincola eggs. Journal of Parasitology 53:694-699.

Philip, C. B. 1955. There's always something new under the "parasitological" sun (the unique story of helminth-borne salmon poisoning disease). Journal of Parasitology 41:125-148.

_____ 1958. Evidence of masking and latency in the fields of insect and helminth microbiology. In: Symposium: Latency and masking in viral and rickettsial infections, ed. by Duart Walker, et al. Minneapolis, Burgess Publishing Company. p. 88-106.

_____ 1962. Helminths as carriers of microbial disease agents of man and animals. In: Biological transmission of disease agents, ed. by Karl Maramorosch. New York, Academic Press. p. 159-169.

_____ W. J. Hadlow and L. E. Hughes. 1954a. Studies on salmon poisoning disease of canines. I. The rickettsial relationships and pathogenicity of Neorickettsia helminthoeca. Experimental Parasitology 3:336-350.

_____ L. E. Hughes, B. Locker and W. J. Hadlow. 1954b. Salmon poisoning disease of canines. II. Further observations on etiologic agent. Proceedings of the Society of Experimental Biology and Medicine 87:397-400.

Simms, B. T., C. R. Donham and J. N. Shaw. 1931. Salmon poisoning. American Journal of Hygiene 13:363-391.

_____ A. M. McCapes and O. H. Muth. 1932. Salmon poisoning: transmission and immunization experiments. Journal of the American Veterinary Medical Association 81:26-36.

Simms, B. T. and O. H. Muth. 1933. Salmon poisoning: transmission and immunization studies. In: Proceedings of the 5th Pacific Science Congress, Vancouver, British Columbia. p. 2949-2960.

U. S. Armed Forces. Institute of Pathology. 1957. Manual of histologic and special staining techniques. Washington, D. C. , United States Government Printing Office. p. 113-114.

APPENDIX

IDENTIFICATION OF THE RICKETTSIA
IN INFECTED CANINE TISSUES

INTRODUCTION

The etiologic agent of "salmon poisoning" disease has not been completely characterized. It is reported to be a "rickettsia-like" organism found as intracytoplasmic bodies in cells of lymph nodes, spleen, and intestines of canids sick with the disease (Cordy and Gorham, 1950). The agent has not been isolated, or shown to be the specific antigen responsible for producing immunity to reinfection. It has not been seen in circulating blood cells of infected canids, and its presence there is indicated only by injection (IP) of whole blood from a dog with SPD into a susceptible dog. Structures believed to be the disease agent were seen in the shell gland of adult flukes, by Hadlow (Philip, 1958). All other evidence for its presence in other stages of the flukes has come from dog transmission studies (Simms et al., 1931; Simms et al., 1932; Simms and Muth, 1933; Philip et al., 1954b).

The purpose of this project was to find a reliable diagnostic method for detecting N. helminthoeca in infected canine tissues.

STAINING TECHNIQUES

Impression smears of lymph node and spleen tissue and smears of whole blood, were prepared from experimentally infected and control dogs. Impression smears were stained using the May-Grunwald stain, Giménez Rickettsial stain, Machiavello's stain, acridine orange fluorescent stain, and the fluorescent tagged antibody technique (FTA), while whole blood smears were stained with the latter two. The procedures used with these techniques are given below. Results obtained are presented in a separate section at the end of the Appendix.

May-Grunwald Stain
(U. S. Armed Forces. Institute of Pathology, 1957)

Materials

Stock Jenner

Jenner's dye	1 gm
Absolute Ethanol	400 ml

Working Jenner

Stock Jenner	25 ml
Distilled water	25 ml

Stock Giemsa

Giemsa powder	1 gm
Glycerin	66 ml

Absolute Methanol	66 ml

Working Giemsa (make fresh; do not reuse)

Stock Giemsa	50 drops
Distilled water	50 drops

Mix the glycerin and Giemsa powder. Place the mixture in a 60 C oven for 30 minutes to two hours, then add 66 ml of 100 percent Methanol.

Staining Procedures

1. Fix in 100 percent Methanol for three to five minutes.

2. Stain in working Jenner for five to six minutes.

3. Transfer directly into working Giemsa for 45 minutes.

4. Rinse quickly in distilled water.

5. Blot or air dry and examine.

Rickettsial bodies and cell nuclei stain deep purple.

Giménez Rickettsial Stain (Giménez, 1964)

Materials

Stock Solution of Carbol Basic Fuchsin

Basic Fuchsin	10 gm
Ethanol (95 percent)	100 ml
Aqueous phenol	4 ml
Distilled water	250 ml

Add the above two to 650 ml distilled water. Keep 48 hours

at 37 C before using.

Sodium Phosphate Buffer Solution (pH 7.45)

0.2 M NaH_2PO_4	3.5 ml
0.2 M Na_2HPO_4	15.5 ml
Distilled water	19.0 ml

Working Carbol Basic Fuchsin

Stock solution	4 ml
Buffer; pH 7.45	10 ml

Other Solutions Used

0.8 percent aqueous malachite green oxalate

Staining Procedure

A very thin smear is made of the tissue to be stained. After

air drying, and either with or without fixation by passing through a

flame, the smear is covered with carbol basic fuchsin (working

solution) and let stand one to two minutes. After thorough washing

in tap water, the smear is covered with malachite green solution

for six to nine seconds, washed with tap water, covered again with

malachite green for six to nine seconds, again washed in tap water,

and the slide dried and examined with the microscope.

Rickettsial bodies stain strong red, cells greenish blue, and

the background slightly green.

Machiavello's Stain (Lillie, 1965)

Materials

Dissolve 0.25 gm basic fuchsin (90 percent dye content) in 100 ml distilled water. Immediately prior to staining, buffer at pH 7.2 - 7.4 with 4 ml of phosphate buffer prepared as follows:

KH_2PO_4 9.08 gm in 1000 cc distilled water

$Na_2HPO_4 \cdot 12\,H_2O$ 11.88 gm in 1000 cc distilled water

Mix in the proportion of 19 parts of the first to 81 parts of the second to obtain a buffer mixture of pH 7.4.

Staining Procedure

1. Flood the slide with 0.25 percent basic fuchsin in phosphate buffer of pH 7.4 and stain for five minutes.

2. Destain for five to ten seconds with 0.5 percent citric acid (see note and caution below).

3. Wash in tap water.

4. Stain for ten seconds in one percent aqueous methylene blue.

5. Wash in tap water, dry and examine under oil immersion.

Rickettsiae stain red, cell nuclei deep blue, and cytoplasm light blue.

Destaining should progress only to the point where the thinner portions of the smear are pinkish-gray in color. Apply the acid for five seconds, wash with tap water and examine the color of the slide by holding it up to the light. Destain for another five seconds if necessary. Two such applications usually suffice. When the stain is satisfactory, the intracytoplasmic bodies appear bright red against a blue background.

Fluorescent Tagged Antibody Technique (FTA)
(Cherry et al., 1960)

Direct Method

This is a relatively new way of detecting antigen-antibody reactions. Its specificity is equivalent to that of conventional serological methods; its sensitivity is often greatly superior. It affords a more rapid means of determining incidence of disease, providing answers sometimes within hours rather than days or even weeks as in some cases with other techniques.

The direct method consists of bringing fluorescent tagged antibodies into contact with antigens fixed on a slide, allowing them to react, washing off the excess antibody and examining the slide by either bright- or dark-field illumination using a U. V. light
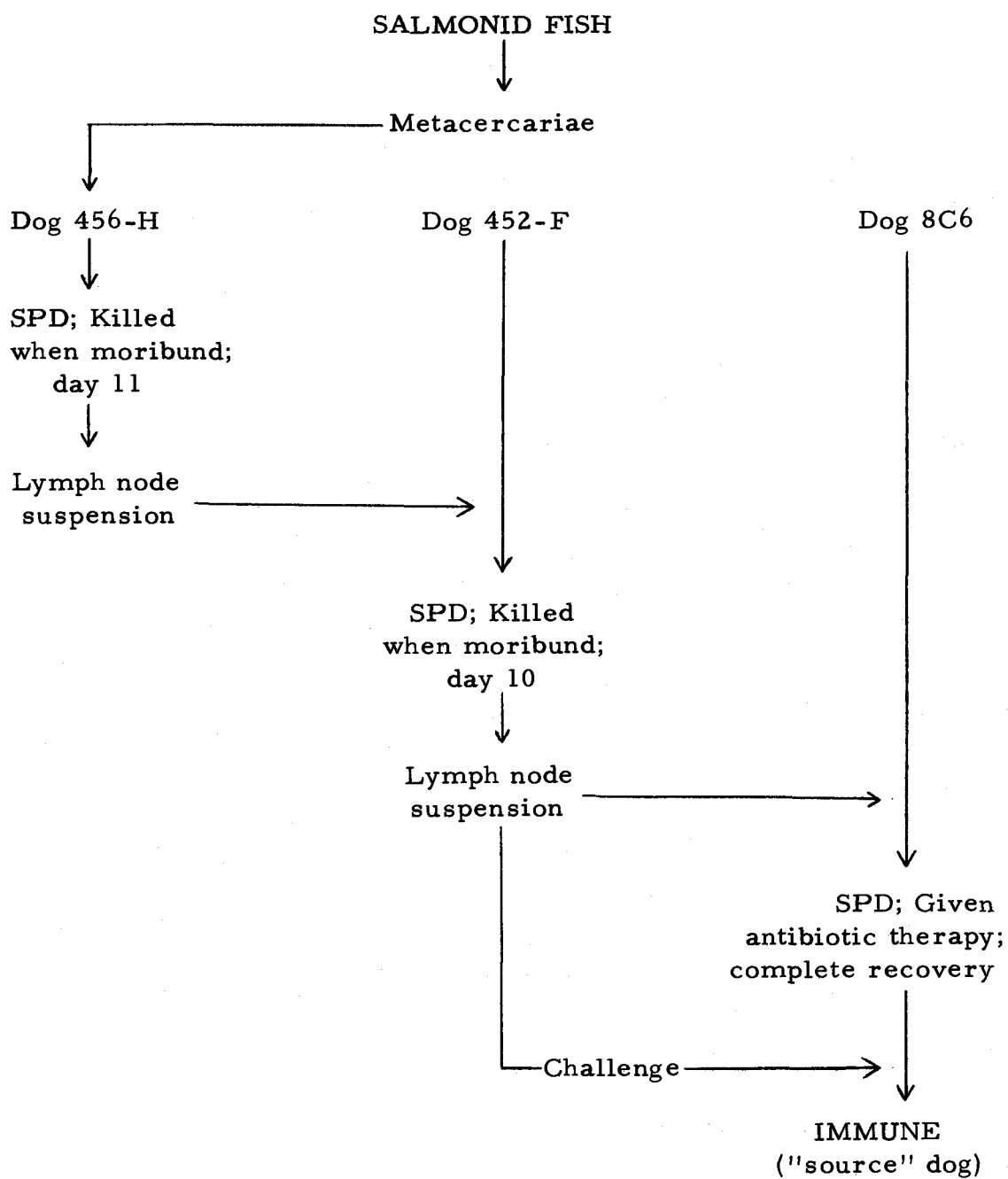
Figure 16. Schema for producing antibody against SPD.

source. The labeled antibodies will be adsorbed onto their homologous antigens and take the form of the antigen particles appearing as fluorescent bodies.

Preparation of Antibody

Dogs used in the preparation of immune serum were six month old Beagles, purchased from the University of California at Davis. The dog (8C6) used as a source of antibody against SPD was immunized as outlined in Figure 16.

Preparation of Conjugate

γ-Globulin was prepared on a diethylamino-ethylcellulose (DEAE) chromatographic column by the method of Levy and Sober (1960). After collection of the γ-globulin from the column, it was labeled with fluorescein isothiocyanate (FITC) using the dialysis technique of Clark and Shepard (1963). γ-Globulin from noninfected control dogs was prepared and labeled similarly with FITC.

Sorption powder was prepared from a mixture of canine liver, kidney, and lymph node tissues, as outlined in U.S. Public Health Service Bulletin No. 729 (Cherry et al., 1960). After labeling by dialysis, γ-globulins were sorbed with this powder to reduce nonspecific fluorescence.

Fluorescent Staining for SPD Antigen

Lymph node impression smears from SPD infected and control dogs were stained using the direct method as outlined in Appendix G of U.S. Public Health Service Bulletin No. 729 (Cherry et al., 1960). Glass slides were marked into two parts, and in one series, lymph node tissue from a noninfected dog was smeared on one side and infected lymph node tissue on the other side. In a second series, infected lymph node tissue was smeared on both sides. Those slides containing noninfected and infected cells, were stained with conjugate prepared from immune serum, whereas the slides containing infected cells only, were stained on one side with conjugate prepared from immune serum, and on the other side with conjugate prepared from nonimmune serum. All slides were fixed in Absolute Ethanol, covered with conjugated γ-globulin (30 minutes to 1.5 hours), rinsed ten minutes or longer in buffered saline, mounted in glycerol saline, and examined using a Zeiss fluorescent microscope.

Acridine Orange Fluorescent Stain
(Livingston and Moore, 1962)

Materials and Staining Procedure

1. Fix cells in Carnoy's for ten minutes.

Absolute Ethanol	60 ml
Chloroform	30 ml

      Glacial acetic acid   10 ml

2. Hydrate through descending ethyl alcohol series to 30 percent.

3. Rinse three times in citrate buffer solution at pH 3.6 - 3.8.

      10.5 gm citric acid monohydric

      15.0 gm $Na_2HPO_4 \cdot 7 H_2O$

      QS to 1 liter

        or

      0.2 M Na_2HPO 71 ml

      0.1 M citric acid   129 ml

4. Stain five minutes with 0.01 percent aqueous acridine orange solution.

      One part 0.1 percent aqueous acridine orange
      plus nine parts citrate buffer at pH 3.8.

5. Rinse three times in citrate buffer for at least five minutes per rinse.

6. Mount in buffer, seal with paraffin and examine with a fluorescent microscope. Ribonucleic acid (RNA) fluoresces orange, deoxyribonucleic acid (DNA) fluoresces green.

RESULTS

The U. S. Public Health Service stain of Giménez (1964) provided an excellent method of identifying the infectious agent. With this stain the organisms stained bright red against a light blue background (Figure 17). The May-Grunwald technique was also satisfactory but provided less contrast since both cell nuclei and rickettsiae stained purple. Machiavello's stain was inconsistent in providing adequate differentiation of the organisms.

With the FTA technique, specific fluorescent spherical and rod-shaped intracytoplasmic bodies were seen in lymph node cells and circulating lymphocytes from infected dogs treated with FITC labeled immune γ-globulin (Figure 18). Similarly treated cells from normal dogs did not contain fluorescent bodies. Likewise, no fluorescent bodies were seen in infected cells stained with nonimmune conjugate.

Larger amounts of cytoplasmic RNA were seen in infected lymph node cells stained with acridine orange than in lymph node cells from control dogs (Figures 19, 20). The reason for this difference is not known; however it may be related to the presence of Neorickettsia helminthoeca. RNA was first seen in circulating lymphocytes four to five days after dogs were infected with lymph node suspensions or metacercariae. The amount of RNA appeared
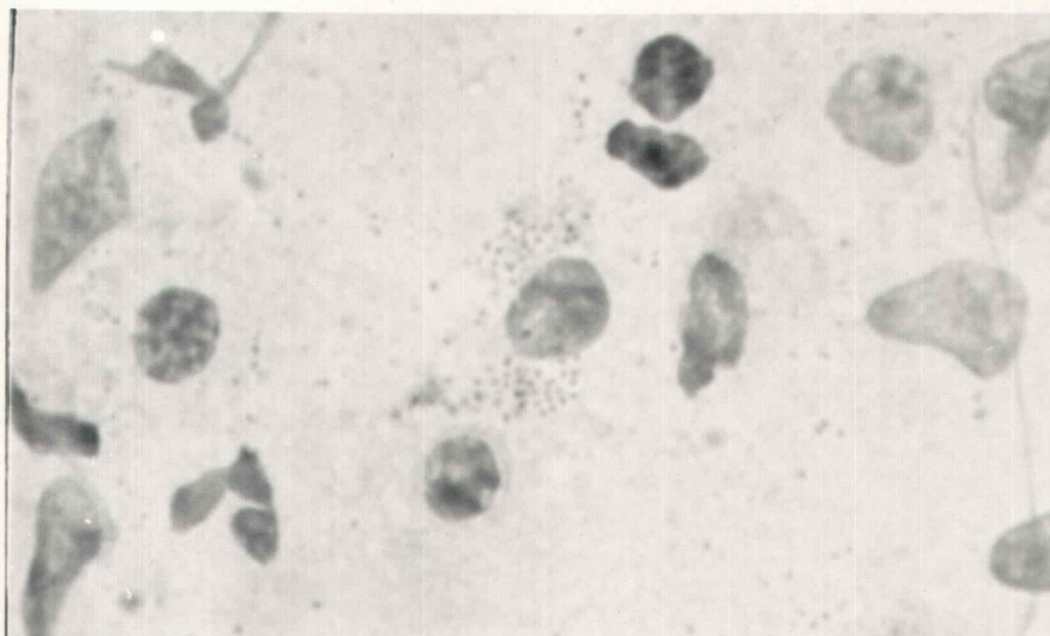
Figure 17. Lymph node impression smear from a dog dead of SPD.
Stained using the technique of Giménez, 1964, X 1600.

Figure 18. Lymph node impression smear from a dog dead of SPD.
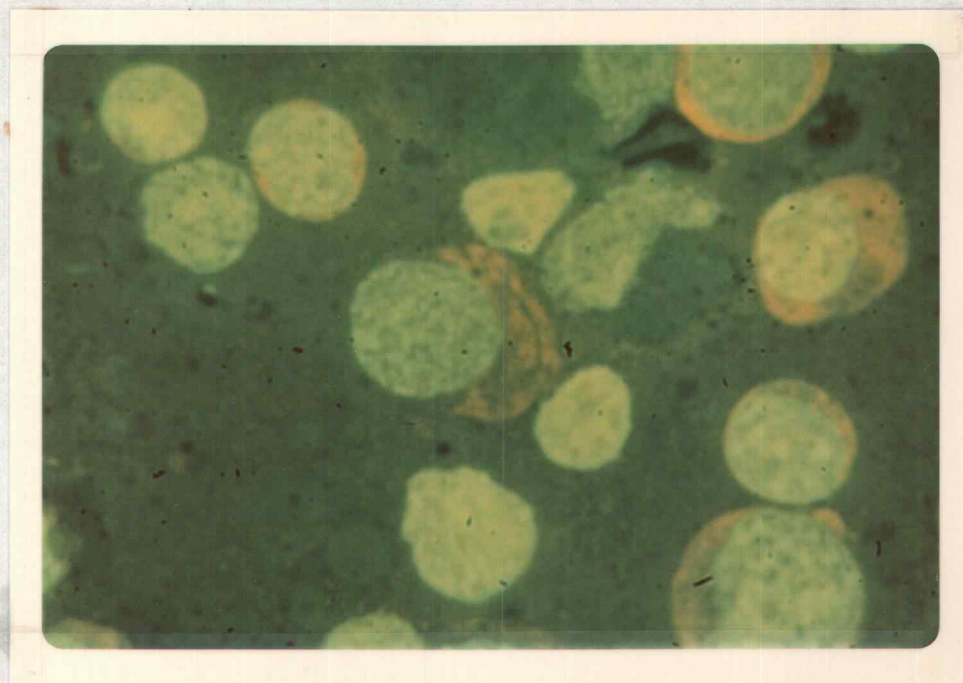Treated with FITC-labeled γ-globulin, X 1600.

Figure 19. Lymph node impression smear from a dog dead
of SPD. Stained with acridine orange fluorescent
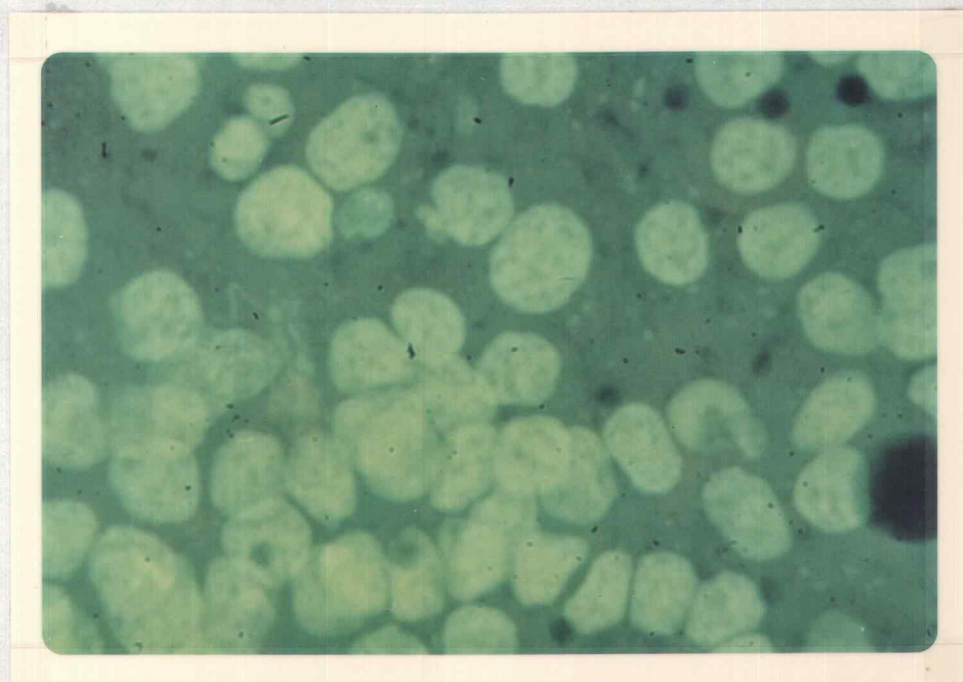stain, X 1600.

Figure 20. Lymph node impression smear from a control
dog. Stained with acridine orange fluorescent
stain, X 1600.

to increase between four and ten days after infection.   No RNA was
seen in lymphocytes before these times or in red blood cells or other
leucocytes.