N. Y., and of a sample of Kidney Antiserum from Dr. J. H. Baxter, Nat. Heart. Inst.

1. Weber, G., MacDonald, H., *Exp. Cell Res.*, 1961, v22, 292.

2. Niemeyer, H. L., Clark-Turri, L., Garces, E., Vergara, F. E., *Arch. Biochem. Biophys.*, 1962, v98, 77.

3. Drabkin, D. L., Marsh, J. B., *J. Biol. Chem.*, 1955, v212, 623.

4. Gutman, A., Shafrir, E., *Am. J. Physiol.*, 1963, v205, 702.

5. Leloir, L. F., Goldenberg, S. H., *J. Biol. Chem.*, 1960, v235, 919.

6. Gutman, A., Shafrir, E., *Am. J. Physiol.*, 1964, in press.

7. Sutherland, E. W., in Colowick, S. P., Kaplan, N. O., (Editors), *Methods in Enzymology*, Academic Press, N. Y., 1955, vI, 215.

8. Good, C. A., Kramer, H., Somogyi, M., *J. Biol. Chem.*, 1933, v100, 435.

9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *ibid.*, 1951, v193, 285.

10. Weber, G., Banerjee, G., Bronstein, S. J., *Am. J. Physiol.*, 1962, v202, 137.

11. Leloir, L. F., Olavarria, J. M., Goldemberg, S. H., Carminatti, H., *Arch. Biochem. Biophys.*, 1959, v81, 508.

12. Villar-Palasi, C., Larner, J., *ibid.*, 1961, v94, 436.

13. Steiner, D. F., Rauda, V., Williams, R. H., *J. Biol. Chem.*, 1961, v236, 299.

14. Marsh, J. B., Drabkin, D. L., *Metabolism*, 1960, v9, 946.

15. Hofert, J. F., Boutwell, R. K., *Arch. Biochem. Biophys.*, 1963, v103, 338.

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Detection of *Toxacara canis* Antibodies with the Fluorescent Antibody Technique.* (29554)

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Recent reports describe the use of the indirect fluorescent antibody (FA) technique in the serodiagnosis of schistosomiasis, filariasis, and trichinosis. Sadun(1) found that, while viable *Trichinella* larvae could not be stained with fluorescein-labeled antiserum, the cuticle of larvae fixed with formalin stains readily. He demonstrated that this is a specific antigen-antibody reaction.

This report describes an attempt to use formalin-fixed second stage *Toxacara canis* larvae in the indirect FA technique to detect *Toxacara* antibodies.

Materials and methods. Four groups of sera were tested. Group A consisted of sera from 4 rabbits and 2 monkeys infected with embryonated eggs of *T. canis* and 2 rabbits and 2 monkeys infected with embryonated eggs of *Ascaris suum* from swine. Embrynated eggs containing second stage larvae were administered by stomach tube.

Group B was made up of sera from 8 rabbits inoculated with "Quadrigen,"† a vaccine containing diphtheria and tetanus toxoids, pertussis vaccine and aluminum phosphate adsorbed and poliomyelitis vaccine, 2 pools of rabbit antisera representing 11 serotypes of enteropathogenic *Escherichia coli*, one rabbit inoculated with *Vibrio cholerae*, 3 lots of commercial normal rabbit serum, one rabbit superinfected with *Trichinella spiralis* larvae, and 5 monkeys infected with polio virus.

Group C included sera from 25 children, age 19-22 months, from a study of "Quadrigen" vaccine. Group D included 49 normal adult blood donors.

Specific anti-human, anti-rabbit, and anti-monkey globulins conjugated with fluorescein

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isothiocyanate were obtained commercially. These globulins were refractionated using a DEAE cellulose column by the method of Riggs *et al*(2). The 0.125 M NaCl fraction was used in this study. Each globulin was titrated against normal and immune sera and the dilution giving the most intense fluorescence without nonspecific staining was used.

Toxacara canis adults were obtained from puppies autopsied at the local animal shelter and *Ascaris suum* adults were obtained from swine at a local abattoir. Species identification was confirmed under a dissecting microscope. Eggs were collected by placing adult females in saline at 37°C overnight or by dissecting out the uterus and mincing with scissors. They were examined microscopically for fertility and to avoid inclusion of eggs from *Toxascaris leonina*. Eggs used to infect animals, for production of immune sera, were incubated in 0.5% formalin at room temperature for 3 weeks before use. They were washed in tap water to remove the formalin prior to administration. The handling of eggs and larvae was greatly facilitated by coating all glassware with a commercial silicone preparation.

Larvae were obtained by embryonating and hatching eggs by the Fairbairn technique as described by Hass and Todd(3). The viable larvae were separated from nonviable larvae, egg membranes, and other debris by covering the mouth of the 50 ml flask in which they were hatched with fine mesh cloth and inverting the flask into a 40 ml centrifuge tube filled with phosphate buffered saline, pH 7.4 (PBS). After an hour or less at 37°C, the active larvae were found in the centrifuge tube, the debris remaining in the flask.

After hatching, the larvae were washed twice in PBS, resuspended in 2 ml of PBS, and immobilized by freezing in alcohol and dry ice. An equal volume of 20% formalin containing 0.5% crystalline bovine serum albumin was added and thawing hastened by immersion for a few seconds in warm water. The larvae remained in contact with the fixative for at least 30 minutes before use. They were then washed 3 times and resuspended in PBS to a concentration of 2000 to 6000/ml. If the larvae were not immobilized before fixation, they coiled tightly upon contact with the formalin, making the test difficult to read.

The procedure for testing for antibody against *T. canis* followed closely that described by Sadun for diagnosis of trichinosis (1).

All sera were inactivated by heating for 30 minutes at 56°C and were tested at a 1:4 dilution. Serum diluted with PBS was mixed in conical 12 ml centrifuge tubes, and 0.05 ml of larval suspension was added and mixed. The larvae were incubated in serum for 30 minutes at room temperature and then washed twice with PBS. As much of the saline as possible was removed after the second wash, 0.05 to 0.10 ml of the appropriate fluorescein-labeled anti-globulin was added, and the larvae resuspended. After 15 minutes incubation at room temperature the larvae were again washed twice in PBS.

A drop of buffered glycerin was then added to each tube. The larvae, in glycerin, were transferred to a microscope slide with a capillary pipette and covered with a coverslip for observation under a fluorescence microscope.

The tests were read under a Zeiss binocular microscope with an Osram HBO 200 mercury vapor lamp as light source, a BG 12 excitor filter, and UG 4 and GG 4 barrier filters.

Results. The techniques described for hatching the larvae and separating them from the debris proved to be quite satisfactory. However, if the eggs were not embryonated for more than 21 days, many larvae had not completed the first molt. The loose cuticle apparently entraps serum, giving a bright fluorescence even with normal serum. Larvae with a very loose cuticle are easily identified, but those in an earlier stage of molting are not so easily distinguished from larvae giving a true reaction.

Although the indirect FA technique in tubes involves a number of steps, it is not difficult to perform and can be completed in about 90 minutes. Under the fluorescence

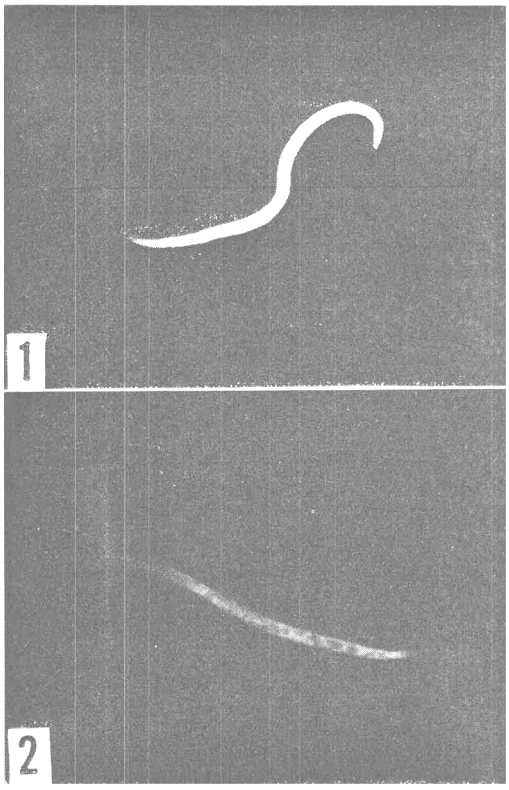

FIG. 1. Indirect staining of *Toxacara canis* larva with immune serum.

FIG. 2. Indirect staining of *Toxacara canis* larva with normal serum.

microscope, larvae exposed to normal serum or no serum, followed by the appropriate labeled anti-globulin, had a bright yellowish fluorescence, but when larvae were exposed to immune serum, followed by labeled anti-globulin, the cuticle fluoresced a bright green. The difference between a positive and negative serum was quite distinct (Fig. 1, 2). The intensity of fluorescence was graded from 0 to 4+.

After storage of the larvae in the fixative for more than one day the intensity of reaction gradually diminished. Larvae which gave reactions of 2+ and 3+ with immune sera when fresh were completely negative with the same sera after 5 days storage at 4°C. Freezing prior to fixation produced some distortion of the larvae but this did not interfere with the test. Some larvae were stored for 2 days at —20°C after freezing in dry ice and alcohol with no deleterious effect.

Results of testing successive bleedings from the monkeys and 4 of the 6 rabbits in Group A are shown in Table I. Preinfection sera from the 6 rabbits and 4 monkeys in this group were negative.

None of the sera in Group B gave a reaction except one lot of commercial rabbit serum which gave a very weak reaction.

Five of the 25 children in Group C (from the "Quadrigen" study) gave reactions varying from + to 3+ and one gave a weak recation (±). Sera from previous bleedings from 12 of these children, including those who gave positive results, were tested with the results as shown in Table II.

In Group D, sera from 17 or 35% of the 49 blood donors gave a reaction of + or

TABLE I. Results of Testing Sera from Infected Animals, Indicating Intensity of Flourescence.

No.	Infecting organism	Intensity of FA reaction at varying weeks from initial infection							
Monkeys*		Weeks → 0	4	8	13	16	29		
85	*T. canis*	—	2+	2+	3+	3+	3+		
86	" "	—	+	+	2+	2+	2+		
87	*A. suum*	—	—	—	—	—	—		
88	" "	—	—	—	±	±	±		
Rabbits†		Weeks → 0	2	3	7	11	14	17	27
2	*T. canis*	—	3+	3+	3+	3+	3+	3+	4+
3	" "	—	4+	4+	4+	3+	4+	4+	4+
4	*A. suum*	—	—	—	—	—	—	+	—
5	" "	—	—	—	—	—	—	—	±

* Monkeys received 15 thousand infective eggs at 0 weeks and 30 thousand infective eggs at 8 weeks.

† Rabbits received 15 thousand infective eggs at 0 weeks and 3 weeks, and 30 thousand infective eggs at 11 weeks.

TABLE II. Results of Testing Successive Serum Samples from Children, Indicating Intensity of Fluorescence.

Identification	Age in months 3-6	7-10	13-16	19-22
KH	—	—	—	—
LW	—	—	—	+
SM	—	—	+	2+
KG	—	—	—	2+
JS	nd*	—	—	3+
TK	—	—	—	2+
MM	—	—	—	—
LG	—	—	—	—
TB	—	—	—	—
DS	—	—	—	—
AT	—	—	—	—
EO	—	—	—	±

* nd = not done.

more, 6 gave a ± reaction, and 26 were negative. These individuals ranged in age from 18-46 years, 43 were males, 6 were females, and all were white. There was no significant difference among 5 year age groups or ABO blood groups when tested by Chi square at the 5% level.

Discussion. Results from the first group of sera indicate that the indirect FA technique behaves in the same manner with *T. canis* larvae as it does with the larvae of *Trichinella spiralis*(1).

Although no clinical signs of infection were observed in the infected animals, except perhaps poor weight gains in the rabbits, at autopsy the livers of these animals were studded with lesions not more than a few millimeters apart in any part of the organ. Results from Group A show that antibodies to *T. canis* can be detected within 2 weeks of infection. Cross reaction with sera from animals infected with *A. suum* were questionable or weak. They did not appear for 13 weeks and after 2 doses of infective eggs in the monkey, and not until 17 weeks and 3 doses of infective eggs in the rabbits. These reactions were weak and the intensity of reaction did not show any increase 16 weeks after its appearance in the monkey.

The results from Group B indicate that there is no cross reaction with a number of antigens to which children are commonly exposed.

Twenty per cent of the children in Group C reacted to this test. This might be explained as a cross reaction with antibodies to antigens other than *T. canis*. The only significant cross reaction with *Toxacara* antigens that has been reported, however, is with *Ascaris* and in this study anti-*Ascaris* serum from infected animals gave only a weak reaction which did not increase during 16 weeks after its appearance.

It cannot be assumed that the results from this small, non-random group of children estimate the prevalence of *Toxacara* antibodies. The children tested are, however, within the age range of reported clinical cases—over half of Snyder's cases(4) were between 16 and 24 months—and the epidemiology of this infection suggests that there are many subclinical infections. The age at which positive reactions appeared also coincides with clinical and epidemiological findings in other studies.

The 35% rate of positive reactions among the adult blood donors in Group D must represent a cross reaction, a persistence of antibody acquired in childhood, or both.

Summary. The indirect fluorescent antibody technique was used to detect antibody against *Toxacara canis* in 4 groups of sera. In a group of animals infected with *T. canis* or *A. suum*, *T. canis* antibodies appeared within 2 weeks of infection and a weak reaction with anti-*Ascaris* sera appeared after repeated infection. Sera from a group of rabbits and monkeys inoculated or infected with various bacterial, viral, and parasitic antigens gave no significant reaction. Five of 25 children, age 19 to 22 months, and 17 of 49 normal adult blood donors gave a significant reaction.

1. Sadun, E. H., *Exp. Parasitol.*, 1963, v13, 72.
2. Riggs, J. L., Loh, P. C., Eveland, W. C., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 655.
3. Hass, D. K., Todd, A. C., *Am. J. Vet. Res.*, 1962, v23, 169.
4. Snyder, C. H., *Pediatrics*, 1961, v28, 85.

Received June 7, 1964. P.S.E.B.M., 1964, v117.