The Use of an Improved Fluorescent Antibody Procedure in the Demonstration of Leptospira in Animal Tissues

by G. Maestrone*

SUMMARY

Increased sensitivity of the fluorescent antibody procedure was achieved by modification of the method employed. Specimens collected from experimentally infected laboratory animals and naturally infected domestic and wild animals were examined by means of this method. The results compared very favorably with those obtained by other investigators who used serological, cultural, animal inoculation and silver impregnation procedures on the same specimens.

The results obtained from extensive application of immunofluorescence to the detection of leptospira in chicken embryo specimens suggested certain modifications in the methodology previously described (1) which remarkably improved the sensitivity of the method.

Since the fluorescent antibody (FA) procedure has been applied in a limited scale to the diagnosis of leptospirosis (1-2-3-4-5-6) this improved method was compared with isolation, serological and histological procedures in the detection of leptospira in specimens collected from different animal species.

Materials and Methods

Specimens: The materials used in this study, except for those of dog origin, were received from investigators working in different areas of the country†. Table 1 lists the source of specimens as well as the time elapsed between the collection and the processing of the specimens and between the processing and the application of the FA procedure. Specimens were submitted either as formalin fixed blocks or as air dried tissue smears, with formalin-fixed kidneys making up the majority of the samples. A few tissues, originally fixed in formalin, were shipped in 70% ethanol.

Fluorescent Conjugates: *Anti-Leptospira canicola, L. ictero-haemorrhagiae* and *L. pomona,* immune sera, globulins and conjugates were prepared according to the procedure outlined previously (1). Sera and globulins had an agglutination-lysis titer of 1:6400 against the homologus antigens. The conjugates, when ready for use, had a titer of 1:400, reflecting the fact that conjugation with fluorescein required dilution of the protein concentration of the globulin to 1%, and that absorption with wetted extracts of tissues produced a further decrease in antibody level.

Tissue Extracts: Acetone extracts (1) were prepared from non-infected, fresh and formalin-fixed dog, cat, and rabbit organs and whole chicken embryos. Two absorptions were sufficient to remove all the non-specific fluorescence when the conjugates were applied to specimens homologous with the extract used for absorption, but not when the conjugates were applied to heterologous samples. Gelatin absorbed conjugates were used to stain sections of

*From the Animal Medical Center, New York, N.Y. Dr. Maestrone's present address is: Chemotherapy Department, The Squibb Institute for Medical Research, New Brunswick, New Jersey.
This work was partially supported by the National Institutes of Health (Grant No. E-2181), Bethesda, Maryland.

†The author wishes to express his gratitude to the following investigators for supplying specimens and related serological, isolation and histological data.

Dr. P. Bennett, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa; Dr. L. Clark, Leptospirosis Laboratory, University of Pennsylvania, New Bolton Center, Kennett Square, Penna.; Mrs. M. M. Galton, Veterinary Public Health Laboratory, Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Ga.; Dr. A. Morter, Leptospira Laboratory, Purdue University, West Lafayette, Indiana; Mr. J. Reilly, New York State Conservation Department; Griffin Laboratory, Slingerlands, New York; Dr. E. Roth, Department of Veterinary Science, Louisiana State University, Baton Rouge, Louisiana, Dr. R. Smith, Department of Veterinary Science, University of Massachusetts, Amherst, Massachusetts.

TABLE I. Specimens Examined by Means of the Fluorescent Antibody Procedure

Species	Animals Examined	Specimens	Time Between Collection* & Preparation**	Time Between Preparation and Processing (i.e. Treatment with Fluorescent Conjugate) Tissue Smears	Formalin Fixed
			Hours	Days***	Days
Dog	9	Kidneys	2	1-3	30-300
Guinea Pig	2	Kidneys	1		45
Hamster	25	Various Tissues	1		6-50
Bovine Adults	21	Urines	Unknown		4-6
Fetuses	10	Various Tissues	1-10	2-20	15-30
Equine Fetus	1	Various Tissues	15	15	36
Swine Fetuses	18	Various Tissues	4-36	15-30	17-49
1-7 day old	9	Various Tissues	1-24	16-30	26-49
8-30 day old	4	Kidneys	1	21-27	26-33
Adults	4	Kidneys	1-2	12-19	11-37
Wild animals	36	Kidneys	3-24		30-450
Total	139		1-36	1-30	4-450

*Collection means either time of death or time of abortion.
**Preparation means either making of smears from tissues and fixation of tissue blocks in formalin or addition of formalin to urine specimens.
***At room temperature, for most of the specimens in the summer.

formalin-fixed tissues, which were mounted on slides with gelatin.

Fluorescent Microscopes: A "Fluorex" (Reichert) apparatus, which uses an Osram HB200 Lamp, and a microscope equipped with darkfield condenser and with magnifications of 200, 450, and 600X were used. The filter combination UG1 (Reichert) and Wratten 2A (Kodak) was used most frequently; the combination S-GB 12 (Reichert) and GG-9 (Reichert) was used for low fluorescence specimens and for photomicrography.

Application of the FA Technique: The preparation of smears and sections followed the procedure already described (1). Slides of fresh material were fixed in acetone for five minutes, dried in an incubator at 37C for five minutes, washed in pH 7.2 phosphate buffered saline containing 3% Tween 80* for 10 minutes, blotted with filter paper and reacted with the fluorescent conjugate for 40 minutes at room temperature in a moist chamber. After washing for 10 minutes in buffered saline the slides were counterstained with rhodamine non specific conjugate** for one minute, washed for five minutes in buffered saline, mounted in buffered glycerine and covered with a covership. The slides made from formalin-fixed material, before being washed in the Tween 80 saline, were dipped in saline containing 1% ammonium hydroxide*** for three minutes. The conjugates were then applied for two hours at 37C.

When no leptospira were visible in a preparation, after the use of the above procedure, a second treatment of the slide with an anti-rabbit globulin fluorescent conjugate**** was applied for 40 minutes to the slides of fresh specimens and for two hours to those of formalin-fixed specimens. This second treatment frequently revealed the presence of leptospira.

*Ruger Chemical Co., Long Island City, N.Y.
**Bacto-FTA Counterstain-Difco Laboratories.
***Merck Reagent Grade.
****Sylvana Chemical Co., West Orange, New Jersey.

When leptospira were visible, duplicate sections or smears were treated, one with normal rabbit serum, one with immune anti-leptospira globulin before being layered with the immune fluorescent conjugate. Where previously specific staining had taken place, the slides which had been in contact with the immune nonlabelled globulin did not show fluorescence.

Tween 80 was used before the fluorescent conjugate because comparative studies in which this surfactant was applied either before or mixed (7) with the conjugate, demonstrated that a 3% concentration applied before the conjugate improved four-fold the brightness of the stain. The contact of the formalin-fixed specimens with 1% ammonium hydroxide, before application of the conjugate, made the detection of leptospira possible, confirming preliminary results (1). Rhodamine counterstain applied after the immune conjugate eliminated completely the background fluorescence and gave a much better contrast than when added to the immune conjugate (8).

An anti-rabbit globulin conjugate applied to the slides already processed with an anti-leptospira conjugate of rabbit origin, demonstrated the presence of microorganisms in several specimens which otherwise would have been diagnosed as negative.

Results

Guinea Pig Specimens: Formalin fixed kidneys from 2 animals experimentally infected with an unknown strain of leptospira were examined and found positive.

Dog Specimens: Kidney sections from 9 serologically positive animals, in which leptospira had been demonstrated by means of the FA procedure a short time after death, were examined after fixation in 10% formalin. The results obtained were identical to those obtained from freshly frozen tissues.

Hamster Specimens: Formalin-fixed tissues from 11 hamsters, experimentally infected from strains of *L. pomona* (5 animals) *L. ballum* (2 animals), *L. batavia* (1 animal) and an unknown strain (3 animals) and sacrificed from 3 to 17 days post-infection were examined. Dark-field microscopy had shown leptospira in the livers of all animals. Micro-organisms were visible by means of the FA procedure in the brain, liver, spleen, lung, muscle, and testicle of all animals. Leptospiroemia was present in all animals except two. Kidney, spleen, liver and lung contained the highest number of micro-organisms; muscle, testicle, brain and blood of the heart contained a much smaller number. Formalin-fixed kidneys collected from 12 hamsters inoculated 18 days earlier with urines from suspected cows (8 animals) or with water from a stream running in an infected area (4 animals) were FA positive in all but one case. Very few leptospira were demonstrated in these specimens, which may explain the negative results obtained by the application of dark-field microscopy and cultural examinations to the same specimens.

TABLE II. Results Obtained from the Application of the Fluorescent Antibody Procedure to the Detection of Leptospira in Tissues of Bovine Fetuses

Organ	FA Positive	FA Negative
Kidney	5	1
Liver	4	2
Stomach	4	0
Peritoneal Fluid	1	0
Heart	1	1
Lung	1	0
Intestine	1	0
Placenta	1	0

Equine Specimens: Smears and formalin-fixed samples from kidney and liver of a fetus aborted from a mare with negative serology were examined and found negative for leptospira.

Bovine Specimens: Twenty-one specimens of urine (preserved with 1% formalin) collected from animals with a positive serology against *L. pomona,* (11 animals), *L. grippotyphosa* (8 animals) and *L. batavia* (1 animal), were examined. Four of these animals had aborted a few weeks earlier, but cultures and animal inoculation of the urines had given negative results. Urine from 4 cows with positive serology for *L. pomona* and the one positive for *L. batavia* contained leptospira. Individual microorganisms were clearly visible in most of the specimens.

In a series of 10 aborted fetuses, 7 were diagnosed as positive for leptospirosis. The serology of 7 of the 10 mothers was available. Three cows with positive serology gave 3 positive fetuses; the four cows with negative serology gave 3 positive fetuses and one negative. A selection of tis-

TABLE III. Results Obtained from Application of the Fluorescent Antibody Procedure to the Detection of Leptospira in Specimens of Swine Origin

Animal Age	Number of Animals	Type of Specimens Submitted			FA Results				
		Smears*	Formalin Fixed**	Smears & Formalin	Posit. Total	Kidney	Liver	Stomach	Peritoneal Fluid
Fetuses	18	5	1	12	11	11/18***	8/10	3/3	1/1
1-7 days	9	3	0	6	7	7/9	4/4	1/1	
8-30 days	4	0	0	4	3	3/4			
Adults	4	1	2	1	4	4/4			
Total	35	9	3	23	25	25/35	12/14	4/4	1/1

*Obtained from fresh tissue.
**10% formalin-fixed tissue blocks.
***Numerator indicates number of positive results. Denominator indicates number of samples examined.

TABLE IV. Comparative Results Obtained by Serological, Isolation and Fluorescent Antibody Procedures in the Diagnosis of Leptospira Infection of Wild Animals

Species	Number of Animals	Positive Serology	Kidney	
			Positive Isolation	Positive Fluorescent Antibody
Armadillo	2	2	2	2
Beaver	1	1	1	0
Mouse	1	0	1	1
Nutria	2	2	2	2
Opossum	2	2	2	2
Rabbit	1	1	1	1
Raccoon	6	5	3	6
Rat	3	0	3	3
Skunk	4	4	4	4
Woodchuck	1	0	1	1
Deer	1	0	Not done	0
Fox (gray)	3	0	" "	0
Fox (red)	9	4	" "	5
Total	36	21	20	27

sues was examined from the 7 positive fetuses and the results are reported in Table II. The kidneys were found to be very rich in leptospira. The examination of fixed tissues and of fresh smears from the same specimens gave identical results.

Swine Specimens: Tissues collected from 35 animals were examined (18 fetuses, 13 piglets and 4 adults). The data obtained in this study are summarized in Table III. Kidneys were submitted from 21 animals, kidney and liver from 11 animals, kidney and stomach from 2 animals, kidney, liver and stomach from one animal and kidney, liver, stomach and peritoneal fluid from one animal. There was a complete correlation in the 23 instances where tissues were submitted both as fresh smears and as formalin-fixed blocks. Identical results were obtained from all specimens of each of the 16 animals for which 2 or more tissues were available. When tissues collected from groups of fetuses or piglets born from the same mother were examined, leptospira were detected in all the animals of 2 groups, in 2/3 of the animals of one group and in ½ of the animals of 2 groups. Serological data were available for all the 4 adult animals (3 positive, 1 negative) and the 18 piglets (all negative). Leptospira infection was demonstrated in all the adult animals and in 11 out of 18 piglets. Silver impregnation results were available for 8 animals; only 6 were positive by means of this procedure against 8 by means of the FA procedure.

Wild Life Specimens: Kidneys collected from 36 animals representing 12 species were examined. Serological data were available for all these animals and isolation data for most of them, as it can be seen in Table IV. Several serotypes were de-

tected both by means of serological and isolation studies. There was a perfect correlation between the results obtained by means of conventional and FA procedures. Leptospira were present in different concentrations ranging from few scattered individuals to large masses.

Discussion

The laboratory methods recommended for the demonstration of leptospira (9-10) are delicate, time consuming, and therefore seldom used on a routine basis by the diagnostic laboratory. Cultural and animal inoculation techniques can be successfully applied only when the specimens contain live microorganisms, a condition which can be seldom accomplished in the case of farm and wild animal specimens. The silver impregnation technique gives positive results when tissues are properly fixed and processed and a large number of organisms are present. In this work the results obtained by means of applying the FA procedure to both fresh and formalin-fixed tissues compare very favorably with those obtained from the same material by means of standard procedures.

It was also seen that when only few organisms were present, as in the hamster tissues and in the bovine urines, the dark-field and cultural examinations were not sensitive enough to detect them.

The main advantage of the FA method is represented by the much shorter time required for the application (few hours in comparison with days and weeks for isolation and histological procedures) and its high sensitivity.

Also very important is the possibility of detecting the antigen in formalin-fixed tissues because it will allow fixing of tissues with formalin as soon as it is collected in the field and holding at room temperature for periods of time up to 18 months before applying the FA procedure.

It should be mentioned that when tissues originally fixed in 10% formalin are shipped in 70% ethanol or imbedded in paraffin, no leptospira can be detected, although observation of smears obtained from the same tissues before fixation reveals the presence of infection. It was found that detection of leptospira was possible by the FA procedure in sections and smears after contact for 30 minutes with 70% ethanol, but not after 90 minutes; higher alcohols and xylene did not modify the "staining" property of the microorganisms.

Conclusions

The FA procedure can be satisfactorily used for the routine detection of natural leptospirosis, provided that:

a. The conjugate contains antibodies against the different serotypes present in the area where it will be employed.
b. The conjugate contains a sufficiently high level of antibodies.
c. The proper absorption and counterstain are used to eliminate non-specific fluorescence.
d. The antigen present in the specimens to be examined is preserved by proper fixation or manipulation.
e. The reagents are checked against proper positive and negative controls.
f. The specificity of positive results is investigated.
g. The appropriate tissues or fluids are examined.

ACKNOWLEDGMENT

The author wishes to express his gratitude for the able technical assistance of Mr. M. Benjaminson, M.S. and Dr. R. Ramirez, D.D.M.

BIBLIOGRAPHY

1. **Coffin, D. L. and Maestrone, G.** Detection of Leptospires by Fluorescent Antibody. Am. Jour. Vet. Res. 23: 159-164, 1962.
2. **Sheldon, W. H.** Leptospiral Antigen Demonstrated by the Fluorescent Antibody Technique in Human Muscle Lesions of L. icterohaemorragiae. Proc. Soc. Exp. Biol. & Med. 84: 165-167, 1953.
3. **Multon, J. E. and Haworth, J. A.** The Demonstration of L. canicola in Hamster Kidney by Means of Fluorescent Antibody. Cornell Vet. 47: 524-532, 1957.
4. **White, F. H. and Ristic, M.** Detection of L. pomona in Guinea Pigs and Bovine Urines with Fluorescein-Labelled Antibody. Jour. Inf. Dis. 105: 118-123, 1959.
5. **White, F. H., Stoliker, H. E. and Galton, M. M.** Detection of Leptospires in Naturally Infected Dogs, Using Fluorescein-Labelled Antibody. Jour. Vet. Res. 22: 650-654, 1961.
6. **Boulanger, P. and Robertson, A.** Fluorescein-Labelled Antibody Technique for the Demonstration of Leptospira pomona. Can. J. Comp. Med. Vet. Sci. 25: 299-306, 1961.
7. **Deacon, W. E., Freeman, E. H. and Harris, A.** Fluorescent Treponemal Antibody Test. Modification Based on Quantitation. Proc. Soc. Exp. Biol. & Med. 103: 827-829, 1960.
8. **Smith, C. W., Marshall, J. D. and Eveland, W. C.** The Use of a Contrasting Fluorescent Dye as a Counterstain in Fixed Tissue Preparations. Proc. Soc. Exp. Biol. & Med. 102: 179-181, 1959.
9. **Babudieri, B.** Laboratory Diagnosis of Leptospirosis. Bull. WHO 24: 45-58, 1961.
10. **Galton, M. M., Menges, R. W. and Shotts, E. B.** Leptospirosis Methods in Laboratory Diagnosis. U. S. Dept. of Health, Education & Welfare, Public Health Service, 1960, CDC, Atlanta, Ga.