This paper describes a study to evaluate the use of fluorescent tagged antibody in the diagnosis of rabies. The authors believe that the FRA test is a valuable addition for the rabies diagnostician, but they also indicate some limitations of the technic.

RABIES DIAGNOSIS BY FLUORESCENT ANTIBODY

I—ITS EVALUATION IN A PUBLIC HEALTH LABORATORY

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IN 1958, Goldwasser and Kissling reported[1] that fluorescent antibody technics could be used to stain street and fixed rabies viral antigens in the brain tissue of experimentally infected mice. They also indicated that this technic might prove useful for the identification of rabies antigens in the brains of animals which were Negri negative. On the basis of this study and more recent work by Goldwasser and Kissling, et al.,[3] the Florida State Board of Health undertook to evaluate fluorescent antibody technics as applied to the routine laboratory diagnosis of rabies. The following report represents the results of routine examination of specimens received at the central laboratory during a 13-month period.

Design of the Study

In the study, Sellers' stained smears prepared from all fresh or frozen brains submitted to the laboratory were examined microscopically for Negri bodies. Smears were also examined for the presence of rabies virus or viral antigen by the Fluorescent Rabies Antibody* (FRA) test. Standard mouse inoculation tests were performed on all specimens and, for the purposes of the study, were considered as the definitive criterion of the presence or absence of infection.

* Hereafter referred to in text as FRA test.

To assure objectivity in observation, it was planned that all specimens for FRA examination be run as unknowns. However, the nature of the senior author's responsibilities made it necessary for him to be informed of the Negri body findings in some 10 per cent of the cases. Since the specimens for FRA testing were processed once weekly, and only then in lots of 20 or more, the individual specimens by that time were actually all unknowns. In only one instance, a human rabies case, was the result of the mouse inoculation test known before the FRA test was completed. For reasons which will be explained later, specimens received at the central laboratory preserved in a glycerol-saline medium were handled differently than those received in a fresh or frozen state.

Materials and Methods

Source of Reagents

Antibodies for labeling were crude globulin solutions prepared from hyperimmune antirabies sera of equine origin

(Lederle).* Fluorescein isothiocyanate,[5] secured from two commercial outlets (Baltimore Biological Laboratories and Sylvana Chemical Company),† was used as the labeling material in the production of conjugates. Acetone-dried brain and liver powders for conjugate adsorption and mouse-brain tissue suspensions for use in the control system were prepared in the laboratory according to procedures described elsewhere.[2,3]

Microscopic Equipment

A Reichert† "Fluorex" unit employing an Osram HBO 200 mercury vapor lamp and a Corning 5840 (half-stock thickness) filter served as the light source. A standard monocular Reichert microscope was fitted with a cardioid darkfield condenser. The objectives, complete with funnel stops when needed, were a 10X, 45X, and 97X oil immersion. The 10X ocular was fitted with a Wratten 2-B gelatin filter.

Labeling of Antisera

The globulin solution, containing specific rabies antibody, was diluted to a protein concentration of 2.5 gm per cent in 0.01M KH_2PO_4/K_2HPO_4-buffered saline (p.h. 7.4-7.6) to which was added 0.5M.$Na_2CO_3/NaHCO_3$ buffer to form 10 per cent of the final volume. To this mixture, powdered fluorescein isothiocyanate was slowly added in a ratio of 1 mg fluorescein to 20 mg protein. The mixture was then subjected to overnight mechanical stirring in the cold, followed by dialysis against repeated changes of phosphate-buffered saline until no traces of fluorescein could be detected in the dialyzing fluid when examined under ultraviolet illumination.[3,2,4] In order to remove nonspecific staining properties, the conjugated protein solution, i.e., the tagged antibodies, was further diluted to 1 gm per cent protein and adsorbed with liver and brain tissue powders.[2,4] After tissue adsorption, final dilution with veronal-buffered saline to which had been added bovine-serum albumin served to reduce nonspecific staining reactions as well as to extend the final volume of staining material.

Proof of Conjugate Specificity and Control Systems

After tissue adsorption, the finished conjugates were tested against known positive and negative specimens. Conjugates which did not produce discrete bright staining or viral antigen in known positive specimens, or conjugates from which the nonspecific staining properties were not almost totally eliminated, were discarded as unsatisfactory for use. Moreover, as each conjugate was produced and before it was used in the test system, proof of specificity was sought in several ways. These included: (1) the failure to stain normal brain tissue or brain tissues in which inclusions of diseases other than rabies were demonstrable; (2) the inhibition or partial inhibition of staining by pretreatment of a positive smear with unconjugated antirabies serum; and, finally (3) the complete inhibition of staining by the addition of rabies infected mouse-brain suspension to the conjugate.[4]

This exposure of conjugate (labeled antibody) to rabies-infected tissue (homologous antigen) not only served as proof of the specificity of any given lot of conjugate, but was also used as a control on each unknown specimen. As described by Goldwasser, et al.,[3] a 20 per cent tissue suspension of normal mouse brain (NMB) was prepared. A similar suspension of infected mouse brain (IMB) was made from mice infected intracerebrally with a strain of fixed rabies virus. Five to ten minutes prior to staining smears, aliquots of conjugate were mixed, respectively, with

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* Acknowledgment of companies or trade names does not constitute endorsement of equipment or products.

equal parts of normal mouse-brain suspension and with rabies-infected mousbrain suspension. The mixing of conjugate with rabies-infected tissue suspension completely inhibited the staining of known positive specimens. In contrast, the similar use of normal mouse brain did not produce inhibition of staining.[4,3,1] Thus, when these two solutions were applied to the separate test and control smears of an unknown, a practical, one-step control system was effected. That is, if the unknown was in fact positive, one would expect to note greenish fluorescent particles in the test smear treated with conjugate-normal mouse brain, whereas the control smear treated with conjugate-infected mouse brain should show no fluorescence if the reaction is specific. It is apparent, then, that identical reactions occurring in both test and control were regarded as nonspecific and that, ideally, greenish fluorescent particles should be absent from both the test and control smears of a negative specimen.

Treatment and Processing of Specimens

On receipt of the brain specimens in the laboratory, four slides were prepared for Negri-body examination according to accepted procedures. Representative portions of the brain tissues were then preserved in glycerine for subsequent mouse inoculation. In addition, portions of Ammon's horns and cerebral cortex were placed in dry specimen bottles and held for FRA examination. Occasionally, slides were made immediately for FRA testing; however, it was found more practical to store the specimens either chilled or frozen and to process them weekly. Periodically, specimens for FRA examination were removed from the refrigerator or freezer and allowed to stand briefly at room temperature. Portions of the tissues were removed from each bottle and two very thin impression smears were made on each of four slides. The smears were allowed to airdry for 30 minutes and then fixed by a four-hour immersion in acetone held at minus 20° C. After that, the racks were removed from the acetone and the smears were thoroughly dried while remaining in the freezer. The slides could then be stained and examined immediately or stored at minus 20° C for several days without impairing staining quality.[3]

Staining of Smears

The direct staining procedure of Coons and Kaplan,[4] as modified by Goldwasser, et al.,[3] was used. Two slides of each specimen were removed from the freezer and the condensed moisture was allowed to evaporate. The two separate smears which had been made on each slide were ringed with a Vaseline-ether solution to prevent mixing of the conjugate. Approximately 0.02-0.04 ml of the conjugate diluted 1:2 with normal mouse brain was applied to the right, or test, smear. An equal amount of the same conjugate mixed in the same proportion with infected mouse brain was applied to the left, or control, smear. Following application of the conjugate-tissue suspensions, the slides were incubated in a moist chamber at 37° C for 30 minutes, after which time the slides were washed ten minutes in two changes of phosphate-buffered saline to remove excess tagged antibody. The slides were taken from the wash, excess moisture was removed, and a drop of buffered glycerol-saline mounting fluid and a cover slip were added.

Reading of Smears

For controls, each group of unknowns was accompanied by a known positive and negative slide. As a check on conjugate specificity, the known negative was read first. To ascertain the sensitivity of the system, the known positive was then examined. As stated previously, with the system working cor-

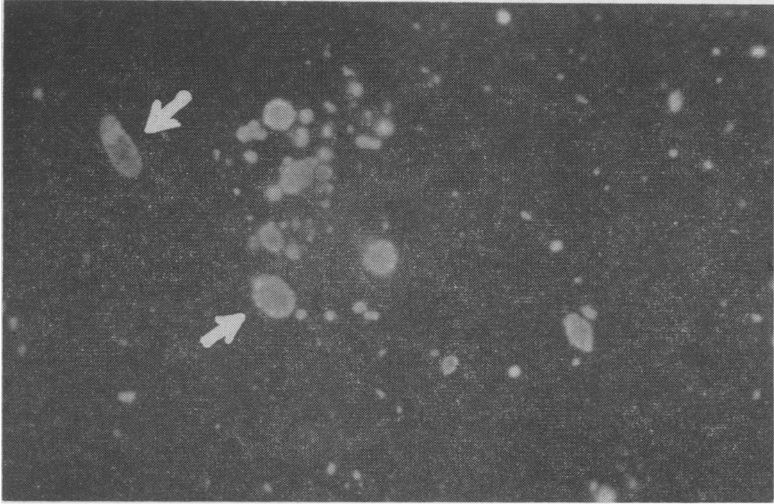

Figure 1—Impression Smear from Brain of Naturally Infected Dog

The fluorescent particles, ranging in size from 0.2 microns to 10 microns, represent aggregates of rabies virus and/or viral antigen. The larger fluorescent particles (arrows) correspond in size to Negri bodies. Magnification X970.

rectly no fluorescence was noted in the negative specimen. In contrast, the known positive test smear (treated with conjugate-normal mouse brain) showed varying numbers of bright green fluorescent bodies ranging in size from the barely visible to those over 20 microns in diameter, whereas, the positive control smear (treated with conjugate-infected mouse brain) exhibited only the blue to greenish-grey autofluorescence of unstained or normal tissue. In reading the unknowns, the test smear was examined first and in the absence of greenish particulate fluorescence the control smear was not consulted. When greenish fluorescent particles were observed in an unknown test smear, they were examined more closely under oil immersion. The control smear of this specimen was then read and any reactions occurring on both sides of the histochemical equation were considered nonspecific.

The aggregates or accumulations of viral antigen, as observed in field specimens, assumed many shapes and sizes. As reported by Goldwasser and Kissling,[1] with few exceptions the larger fluorescent bodies were closely correlated with the size, shape, and distribution of Negri bodies seen on companion smears made from the same specimen and treated with Sellers' stain (Figure 1). It should be emphasized, however, that in every positive specimen the authors observed uncountable numbers of antigen aggregates which were unidentifiable by Sellers' staining, and that even in the absence of the larger fluorescent aggregates, a diagnosis could be rendered on the defined specificity of these smaller fluorescent particles. Such, in fact, was the case with the Negri-negative, virus-positive specimens processed. Although fluorescent structures within the size range of normal Negri bodies were seen on some of these Negri-negative specimens, for the most part the fluorescent particles were less

in number and smaller in size than commonly seen on the typical Negri-positive (Figures 2 and 3).

The phenomenon most frequently observed on positive specimens was the appearance of round fluorescent bodies of varying diameter exhibiting bright peripheral fluorescence with central darkening or mottling. These morphologically distinct "ring-forms" were observed in every positive specimen, occurring with such a high degree of regularity that they were virtually diagnostic (Figure 4).

The nature of nonspecific staining reactions is poorly understood and less well defined. These reactions, occurring even with a highly refined conjugate, might be classified as: (1) granular precipitation of stain, (2) diffuse staining of the tissue, occurring particularly in thick areas of tissue impression smears, and (3) cellular staining of normal tissue components. A conjugate, to be acceptable, must not stain normal tissue cells, and for the most part this undesirable property may be almost totally eliminated by tissue powder adsorption and conjugate dilution. Although the staining reactions caused by the other two factors are usually morphologically distinct and easily differentiated from a specific reaction, it remains the primary function of an adequate control system to identify these nonspecific reactions when they occur.

One should not confuse specific or so-called nonspecific staining reactions with normal tissue fluorescence. Normal tissue fluorescence may be thought of as: a diffuse type of tissue autofluorescence and as normal particulate fluorescence. When smears are properly made and processed, and when proper optical equipment is used for reading the slides, the tissue background will appear dark blue-grey to slightly greenish-grey. Quite often, however, the tissue background will fluoresce intensively, appearing bright blue-grey to white, particularly if the smears are very thick or if the tissue has been stored frozen

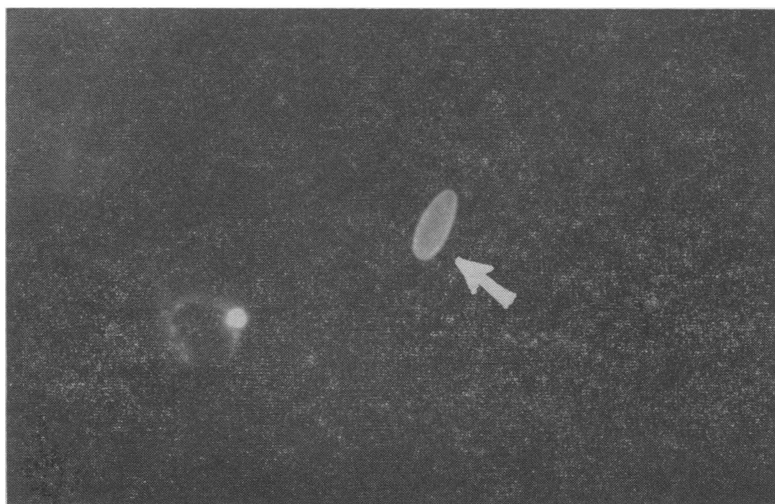

Figure 2—Impression Smear from Brain of Naturally Infected Horse

Arrow points to one large (approximately 6 x 15 microns) antigen aggregate. Although the specimen was Negri negative, several greenish fluorescent bodies within the size range of Negri bodies were seen on FRA examination. Magnification X970.

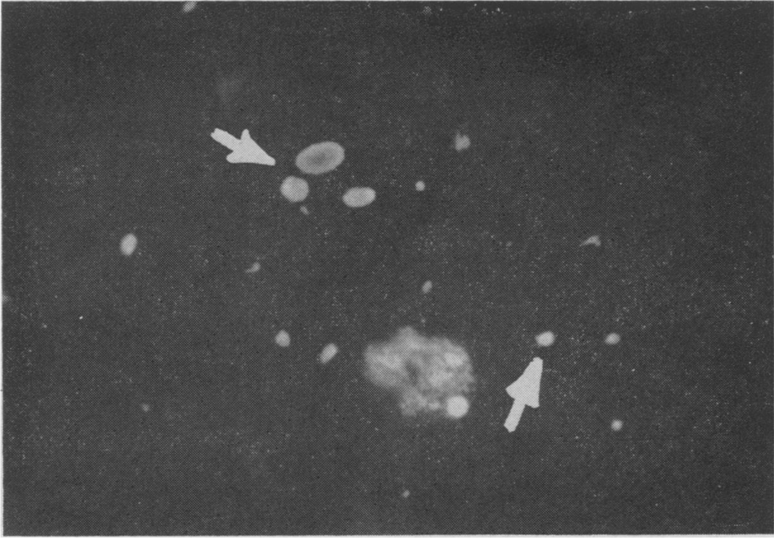

Figure 3—Impression Smear from Brain of Naturally Infected Dog Exhibiting Symptoms of Dumb Rabies

Particles of fluorescent stained antigen (arrows) ranging in size from 1 to 8 microns are much in evidence despite the fact this specimen was Negri negative. Magnification X970.

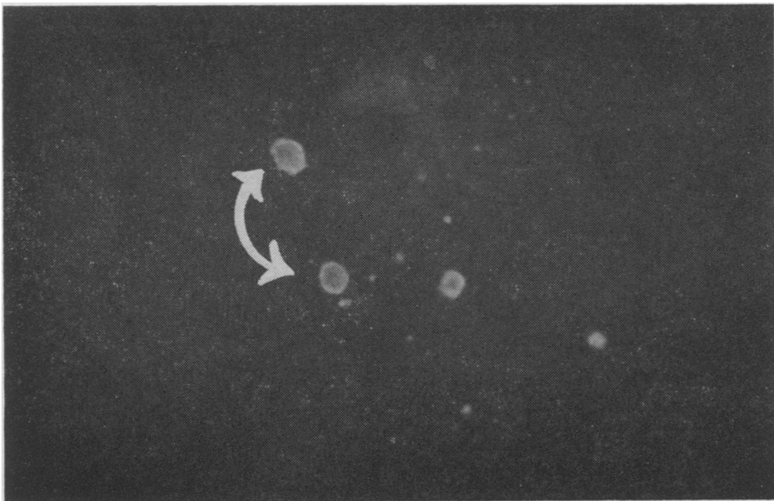

Figure 4—Impression Smear from Brain of Naturally Infected Fox

Arrows point to the "ring-form" of stained antigen aggregates. These round, or nearly round, bodies with bright peripheral fluorescence and central darkening may also be seen on Figures 1 and 3. Magnification X970.

for more than from four to eight weeks before processing. Since the color and intensity of the background fluorescence may be greatly altered by the characteristics of the transmitted light, a filter system for rabies work should be chosen with care. The field (primary) filter should transmit light in the near ultraviolet range, i.e., between 350-400 μ. The eyepiece (secondary) filter should be nearly colorless yet opaque to ultraviolet. Primary filters permitting the transmission of blue-violet light (between 400-450 μ) are to be strictly avoided. Brightly fluorescing yellow, yellow-tan, brown, pink, or white particles may be observed in almost any specimen. This normal particulate fluorescence should be disregarded and not confused with the distinct green fluorescence of attached labeled antibody. It should be obvious, that with the procedures described, it takes a minimum of from seven to eight hours to process any one specimen.

Results and Analysis of Data

Data on each specimen (species; method of preservation; Negri examination results; FRA reading; control system used; results of mouse inoculation; and remarks) were recorded on a specially prepared work sheet. Results of the Negri body examination and mouse test were recorded only after the FRA test was reported as either positive or negative. In reading the slides the accumulated total of FRA positive slides was posted over the total number of slides run on each specimen, e.g., $N^0/2$-or-$P^2/2$.

As seen from Table 1, a total of 825 fresh or frozen specimens was processed. The FRA technic was in complete agreement with mouse inoculations on the total number processed, detecting 70 of 70 positives. No false positive reactions by FRA were recorded. Results of the Negri examination agreed only 98.3 per cent with mouse inoculations. This margin of error was comprised of four specimens which were Negri-negative, virus-positive (Table 1, column 3) and ten specimens which were called Negri-positive but from which no virus could be isolated (Table 2). Review of the Sellers' stained slides of the Negri-positive, virus-negative specimens revealed for the most part only atypical inclusions. In three of these specimens, however, inclusions characteristic of Negri bodies were seen. The FRA examination on these ten brain specimens was negative. In addition, no virus could be isolated from four of this group on which salivary gland tissues were available for examination.

Table 1—Fluorescent Rabies Antibody (Fresh or Frozen Specimens)

1 Species	2 Specimens Examined	3 Specimens Positive		
		Negri Exam.	Mouse Inoc.	FRA
Dog	244	33	35	35
Fox	45	10	10	10
Cat	204	3	3	3
Raccoon	54	7	7	7
Squirrel	63			
Skunk	7	3	4	4
Opossum	27			
Bat	79	5	5	5
Bovine	4	3	3	3
Goat	2			
Monkey	4			
Hog	0			
Bobcat	2	1	1	1
Hamster	12			
Rat	58			
Gopher	1			
Mouse	3			
Rabbit	11			
Horse	2	0	1	1
Human	1	1	1	1
Other	2			
Total	825	66	70	70

Table 2—Fluorescent Rabies Antibody (Fresh or Frozen Specimens)

Species	Specimen Number	Test Results Negri Exam.	FRA	Mouse Inoc.*
Dog	239	+	—	—
Dog	743	+	—	—
Fox	124	+	—	—
Fox	125	+	—	—
Fox	202	+	—	—
Fox	487	+	—	—
Fox	411	+	—	—
Cat	378	+	—	—
Bat	701	+	—	—
Raccoon	380	+	—	—

NOTE: Review of the Sellers stained slides on these specimens revealed for the most part only atypical inclusion bodies. However, on specimens No. 378, 701, and 743 the inclusions were typical of Negri bodies.

* All mice were held for a minimum of 30 days and in five cases blind mouse passage was done in an attempt to isolate virus.
+ = Evidence of virus or Negri bodies.
— = No evidence of virus or viral antigen.

Discussion

Difficulties Encountered and Test Limitations

One of the most distressing hindrances to the routine use of the procedure at this time is the unavailability of commercially produced reagents. Although several good fluorochromes are available commercially, there is at present no dependable commercial source of labeled antiserum. Moreover, it is in the production of a satisfactory conjugate that the major obstacle to successful performance of the test is encountered. It is impossible to predict whether one will secure a suitable conjugate from the combination of any given lot of antiserum and reagent dye. It now appears that both factors may be variable. The authors have succeeded in producing satisfactory conjugates five out of seven times, using six different lots of antisera and four different lots of fluorescein isothiocyanate obtained from two sources. It was apparent that some lots of dye gave better conjugation of the protein than did others.

It should be stressed that the success obtained with the technic may depend to a large extent on the conscientious application of certain prescribed procedures in the preparation of fluorescent conjugates and in the performance of the test. Persons beginning work with the FRA test should gain first-hand experience in the preparation of reagents. It would also appear advisable, before attempting the test on unknowns, to use a conjugate of proven specificity and work with known positive and negative specimens to gain a great deal of experience in reading slides.

During the initial phase of this study, it was noted that when specimens had been preserved in glycerol-saline, it seemed impossible to obtain the quality of staining observed in fresh or frozen specimens. After a glycerinated brain specimen was called negative by FRA test and proven positive by mouse inoculation, it was undertaken to study this problem more thoroughly. A study was done on 53 glycerinated specimens obtained from naturally infected animals of which eight were positive by mouse inoculation. Of the eight positives, four were Negri-negative. The FRA test failed to detect the presence of viral antigen in the glycerinated specimens from one Negri-negative and one Negri-positive animal. Thus, the FRA technic detected only 75 per cent of the eight glycerinated positives. This is to be compared to the complete agreement obtained between the FRA and mouse inoculation tests on 70 positive specimens processed as fresh or frozen. In addition, controlled experiments on the brains of many experimentally infected mice indicated, to the satisfaction of the authors, that specimens preserved in glycerine were completely unsatisfactory for FRA examination. It was demonstrated conclusively that glycerination even for short periods resulted in almost every case in a material and often a diagnostically critical quantitative and

qualitative decrease in visible fluorescence.

Summary

The procedure for staining rabies virus antigens with fluorescent tagged antibody in the brains of naturally infected animals is described, and is represented schematically in Figure 5. The usefulness of this technic for the routine diagnosis of rabies in the public health laboratory, particularly in the case of Negri-negative animals, is emphasized. It has been demonstrated that FRA test may be used to advantage to help define the antigenic nature of atypical or otherwise questionable inclusion bodies observed during direct microscopic examination. It is believed that the FRA test, as it stands today, is a valuable adjunct to the armamentarium of the rabies diagnostician.

The FRA test was in complete agreement with the mouse inoculation results on a total of 825 fresh or frozen specimens, whereas the results of the Negri examinations gave only a 98.3 per cent correlation. The FRA test detected all of the 70 positives included in the

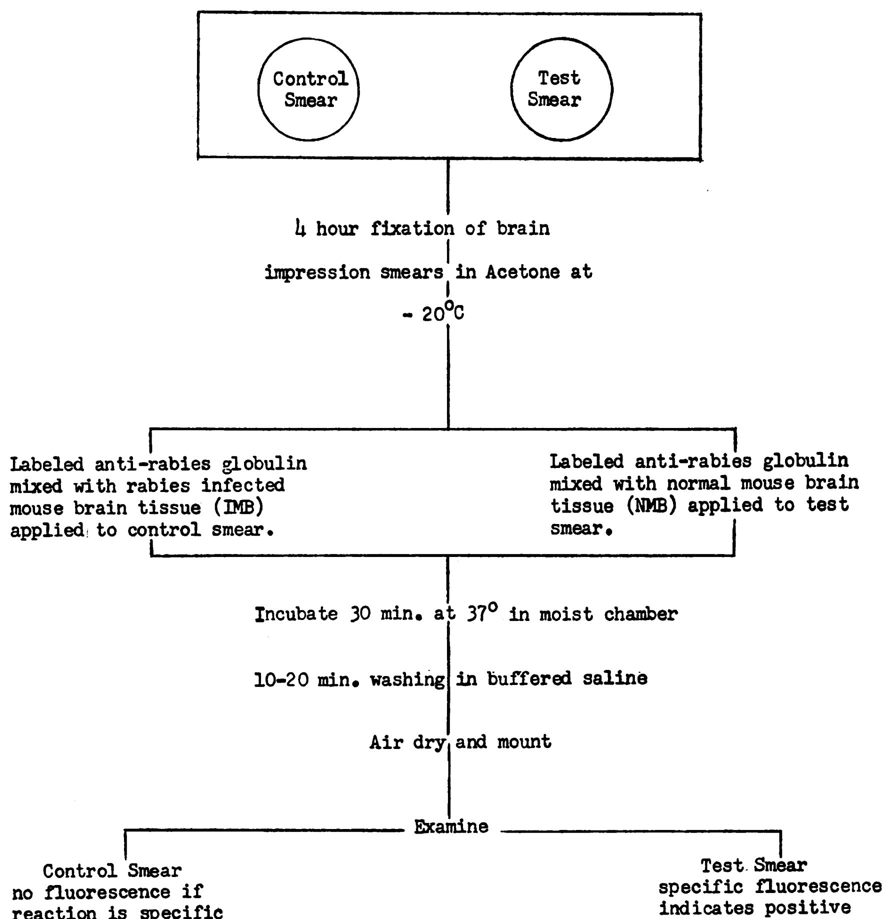

Figure 5—Fluorescent Rabies Antibody Test (Direct Method)

group. In contrast, in only 66 of 70 positives or 94.3 per cent were Negri bodies demonstrable.

Results would indicate that fragments of brain tissue preserved in glycerol-saline are not satisfactory for FRA examination. The factor, or factors, which interferes with the antigen-antibody reaction or "staining" of viral antigen in these specimens is not understood.

Preliminary studies indicate the FRA test to be highly specific. It appears to be more sensitive than the direct microscopic examination of Sellers' stained slides; however, additional studies are needed before any firm conclusions can be drawn on the sensitivity of the FRA test as compared to standard mouse-inoculation technics. It is hoped that, with the application of the FRA test in other laboratories, it will soon be possible to ascertain more precisely the relative merits and limitations of this technic.

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