Efficacy of fluorescent antibody methods for detection of *Pasteurella pestis* in carcasses of albino laboratory mice stored for various periods

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INTRODUCTION

The methods recommended for diagnosis of *Pasteurella pestis* infection in animal tissues include microscopic examination of stained impression smears, bacteriological culture followed by biochemical and bacteriophage tests, animal inoculation, and precipitin tests using soluble plague antigens (Baltzard *et al.* 1956). Bacteriological culture and animal inoculation are sensitive and specific when test materials are well preserved, but these methods seldom give positive results when the specimens are from putrefied or mummified carcasses. In such cases, the modified Ascoli test developed by Larson, Philip, Cicht & Hughes (1951) or the haemagglutination and complement-fixation tests of Chen, Quan & Meyer (1952) and Chen & Meyer (1954) have proved to be of value. Larson reported significant precipitinogen titres in animal tissues stored at 37° C. for periods as long as 14 weeks. Chen reported excellent results with complement fixation tests of plague antigens extracted from decomposed carcasses and from partially desiccated tissues held at 37° C. for 10 days.

A fluorescent antibody (FA) test recently has been developed for plague detection and was used successfully in examinations of fresh tissues containing viable bacteria (Moody & Winter, 1959) and tissues from human cadavers (Cherry, Goldman, Carski & Moody, 1960) and animal carcasses lacking viable bacteria (Kartman, 1960). The test not only possesses the serological specificity shown by Larson's modified Ascoli test, but offers other advantages inherent to the technique; i.e. speed, and the requirement of exceptionally small amounts of test material.

Investigations on the use of fluorescent antibody staining techniques for the identification of various bacteria usually have emphasized the efficiency of the test under conditions designed to obtain maximum preservation of specimens. Few studies have dealt with the efficacy of fluorescent antibody diagnosis of pathogens in decomposed or putrefied specimens. Accordingly, experiments were conducted to compare FA techniques with bacterial cultivation, animal inoculation, and precipitin tests for detection of *P. pestis* in laboratory albino mouse carcasses held under a variety of conditions.

METHODS

Laboratory mice 1–4 months old were inoculated subcutaneously with *P. pestis* (Sackacs strain). These animals were collected on the day of death and an equal number of normal control mice were killed at that time. Additional controls consisted of mice dying after inoculation of *Brucella tularensis* (strain H 8859). Individual carcasses of plague-positive, tularaemia-positive, and normal control mice were first loosely wrapped, then grouped and stored for varying periods at three different temperatures as follows: 4–5° C. (refrigerator), 20–25° C. (room), and 37° C. (incubator).

In addition to the studies on mice, an opportunity presented itself to study tissues removed from two guinea-pigs 7 years previously. These animals had died after inoculation with the Alexander strain of *P. pestis*, and on 9 September 1954, samples consisting of the combined spleen and liver of one, and of spleen, liver, and a portion of lymph node from the second were removed and held in Petri dishes at room temperature and humidity. At the time of the present studies, these tissues had become completely dehydrated and remained as a thin layer of residue on the bottom of their respective Petri dishes. Portions of each sample, as described later, were rehydrated and slides prepared for examination by fluorescent antibody methods.

Preparation of slides for fluorescent antibody staining

At intervals the stored albino mice were randomized and dissected. Impression smears were made of lymph node, spleen, and liver tissues. Smears of bone marrow were made by removing marrow from the opened sections of femurs with sharpened applicator sticks.

As the experiment progressed, frequently it became impossible to discern organ structure. In this case, samples of abdominal and bone contents were taken by loop if liquefied. If the carcasses were severely desiccated it was usually necessary to rehydrate small portions of abdominal contents or bone by grinding with saline in a mortar.

After the slides were prepared and thoroughly air-dried, they were stored unfixed at −40° C. until fluorescent antibody processing was started. The storage period usually was 16–24 hr., although occasionally slides were stored for periods of from 2–3 days apparently with no adverse effects.

Fluorescent antibody staining techniques

All slides were first gently heat-fixed and then stained with rabbit antiplague globulin conjugated with fluorescein isothiocyanate. The specificity of fluorescent antibody staining was controlled by use of the one-step inhibition technique (Goldman, 1961). Procedures for preparation of antisera and fluorescent conjugates, as well as conjugate adsorption techniques, have been reported elsewhere (Kartman, 1960; Hudson, 1961) with the following exception. During the terminal stages of the experiment, the adsorption technique was changed from one utilizing acetone liver powder-celite columns to another using polymerized dextran

gels* according to the techniques described by Zwann (1961) and Curtain (1961) for rapid separation of fluorescent antisera and unconjugated dye. The latter technique was used in processing the mouse carcasses after storage for 340 days at 4–5° C., and for the guinea-pig tissues stored at room temperature in Petri dishes for 7 years.

Numbered slides containing infected material, and control slides, were prepared by one group of workers and then were stained and examined as 'unknowns' by two other persons. On microscopic inspection, slides exhibiting numerous fluorescent bacteria, fluorescent bacterial particles or debris, or extensive areas of fluorescence were graded as positive (+) if definite inhibition of fluorescent staining occurred in the accompanying inhibition control. Just discernible to brilliant specific fluorescence was graded +1 to +4 respectively. Even if very brilliantly stained, the results were regarded as inconclusive (±) when few fluorescent bacteria or particles (less than one per microscopic field at ×300) were visible, since in this case inhibition tests were considered unreliable. Results were regarded as negative (−) in the absence of any discernible specific fluorescence.

Standard diagnostic tests

Bacterial culture, animal inoculation, and the precipitin tests were performed according to recommended procedures (Baltazard *et al.* 1956). For bacterial culture, samples were streaked on blood agar plates, incubated at 28° C. and read at 24, 48 and 72 hr. Questionable cultures were confirmed by inoculation into laboratory mice and by specific bacteriophage tests (Cavanaugh & Quan, 1953).

Triturated specimens from mouse carcasses were inoculated into mice or guinea-pigs by subcutaneous or percutaneous routes. All inoculated animals were held for at least 21 days; those dying during this period were autopsied and examined for typical plague pathology and for the presence of bipolar bacilli in stained smears. When animals exhibited questionable clinical course or pathology their tissues were cultured on blood agar plates for further confirmation. Mice and guinea-pigs surviving the observation period were killed, examined for plague pathology, and regarded as negative if no obvious evidence of infection existed.

Precipitin tests were done according to Larson's modified Ascoli test (Larson *et al.*, 1951). Tissues were extracted with ether-saline solution and after centrifugation the clear supernatant fluids were collected. When clear extracts were not obtained, the fluids were filtered. Tissue extracts were then used in a capillary ring test with standardized or absorbed plague-specific rabbit globulin. Controls for this test consisted of extracts of tissues of mice killed with physical force or by tularaemia infection, as described previously. When control tissue extracts were positive, difficult to read, or doubtful, all the extracts were absorbed with equal volumes of normal rabbit globulin before retesting.

During the course of the work, the choice of various techniques was determined by changing conditions in the materials. When carcasses were relatively fresh and well preserved, they were tested by quantitative bacterial culture. When putre-

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* Sephadex G-25, medium grade. Pharmacia Fine Chemicals Inc., Uppsala. The use of trade names is for identification purposes only and does not constitute endorsement by the U.S. Public Health Service.

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faction of the specimens precluded clear-cut results by bacterial culture, inoculations of mice and guinea-pigs were done to find viable *P. pestis*. At later stages, when other tests failed to reveal viable organisms in the carcasses, mouse inoculation was supplemented with Larson's modified Ascoli test.

<center>RESULTS</center>

Tables 1 and 2 summarize the results of fluorescent antibody tests for *P. pestis* in albino-mouse carcasses stored for various periods at three different temperature ranges. The fluorescent antibody tests are compared with other tests performed on the same specimens. Specific fluorescent staining (and extractable antigen) decays with age. Thus these tests were limited by the conditions of preservation. *P. pestis* antigens were better preserved at 4° C. and 25° C. than at 37° C.

Table 1. *Results of tests for* Pasteurella pestis *and its antigens in albino-mouse carcasses stored at 4°–5° C for various periods*

No. positive for *P. pestis*/total number tested

Carcasses	Days stored	Fluorescent antibody stain	Blood agar plate culture	Animal inoculation	Precipitin test
Died of *Pasteurella*	1	5/5	5/5	—	—
pestis infection	4	5/5	5/5	—	—
	7	5/5	5/5	—	—
	17	5/5	5/5	4/5	—
	34	4/5	3/5	2/5	—
	64	3/5	2/5	1/5	—
	140	4/5	0/5	0/5	1/5
	340	15/16	—	—	10/16
Died of *Br. tularensis*	1–64	0/30	0/30	—	—
infection	140	0/5	—	—	0/3
	340	0/3	—	—	2/3
Killed normal mice	1–64	0/30	0/30	—	—
	140	0/5	—	—	0/2
	340	0/3	—	—	0/3

Refrigerated cadavers, 4° *to* 5° C. (*Table* 1)

By the 17th day of storage, smears of tissues from refrigerated mouse carcasses showed only a slight loss in brilliance of fluorescent staining (Pl. 1, Fig. B). At the same time noticeable lysis of plague bacilli was present in all tissues, the bacteria being preserved best in bone marrow. Deterioration of bacterial morphology was present in increasing amounts in tissues in the following order: Bone marrow, lymph node, spleen, and liver. Smears of liver tissue showed *P. pestis* with ragged, incomplete outlines and fine granular stippling of fluorescent plague antigen in the background. In bone marrow smears the bacteria were brilliantly stained, more or less typical in morphology and size, and with occasional faint fluorescent background stippling due to particles of plague antigen.

Examination of tissues on the 34th, 64th, 140th, and 340th days of storage at 4°–5° C. gave results quite similar to those on the 17th day. Specific organs were

difficult to identify and occasionally were not obtained because of deterioration and drying. Nevertheless, when smears could be taken from identifiable lymph node and bone marrow they stained as brilliantly, and in some cases better, than smears made on the 17th day from these tissues. The processes of autolysis and putrefactive bacterial activity made difficult the distinction between abdominal and thoracic tissues. Thus fluorescent staining of particles and products of *P. pestis* was generally variable.

Table 2. *Results of tests for* Pasteurella pestis *and its antigens in albino-mouse carcasses stored at room temperature and at* 37° C. *for various periods*

		No. positive for *P. pestis*/total no. tested			
Carcasses	Days stored	Fluorescent antibody test	Blood agar plate culture	Animal inoculation	Precipitin test
Died of *P. pestis*—	2	5/5	5/5	—	—
stored at room	4	5/5	5/5	4/5	—
temperature	8	5/5	1/5	5/5	—
	16	5/5	0/5	2/5	5/5
	32	5/5	—	0/5	4/5
	82	4/5	—	—	5/5
Killed normal mice	2 to 8	0/15	0/15	—	—
stored at room	16	0/5	0/5	—	0/2
temperature	32	0/5	—	—	0/5
	82	0/5	—	—	0/2
Died of *P. pestis*—	2	5/5	5/5	—	—
stored at 37° C.	4	5/5	0/5	0/5	—
	8	5/5	0/5	0/5	—
	16	4/5	0/3	0/5	3/5
	32	3/5	—	—	3/5
	82	2/5	—	—	1/5
Killed normal mice	2 to 8	0/15	0/15	—	—
stored at 37° C.	16	0/5	0/3	—	0/2
	32	0/5	—	—	0/5
	82	0/5	—	—	0/5

Room temperature and 37° C. *(Table* 2)

When held at room temperature (20°–25° C.) and at 37° C. mouse carcasses reached an advanced degree of dehydration by the 8th day. Lymph node tissue was very difficult to locate and dissect. The internal organs were barely discernible and bones and surrounding muscle were dry and friable. At this stage, slides were made after tissue samples were rehydrated with saline in small porcelain mortars. When stained with fluorescent antibody the smears showed brilliant fluorescent stippling of sand-like particles over the background with occasional apparently intact plague bacilli (Pl. 1, Fig. C).

From the 8th to the 82nd day smears made from mouse carcasses stored at room temperature and at 37° C. showed no additional features of diagnostic interest when stained with fluorescent antibody conjugate. During this period the intensity of fluorescence in specimens held at 37° C. gradually diminished,

virtually disappearing after the 32nd day. Slides from mice held at room temperature were still yielding brightly fluorescing specimens on the 82nd day of storage.

Additional available specimens were dissected tissues of 2 guinea-pigs that had been held at room temperature for 7 years. This material was a residue consisting of a thin scum on the bottom of the Petri dishes. When scraped from their containers and processed they gave a brilliant specific plague stain showing complete inhibition by unconjugated anti-plague globulin in the one-step inhibition test. Fluorescent antibody staining was in all respects similar to that exhibited in smears of tissues from mouse carcasses stored for 32–82 days at room temperature (Pl. 1, Fig. D).

Other observations

From the 2nd day of the experiment, brilliant masses of yellow, red, and blue autofluorescence were present consistently in tissue smears. However, this autofluorescence was never intense enough to obliterate the characteristic apple-green fluorescence of the fluorescent antibody conjugate used.

After 1–2 weeks of storage, tissues from mouse carcasses tested by blood agar plate culture and animal inoculation gave poor to negative results; refrigerated material was an exception, yielding good results for as long as 34 days. Carcasses stored 16 days at room temperature and 8 days at 37° C. were virtually sterile when cultured on blood agar plates. Thus, at this stage, diagnostic tests which depended on the presence of viable plague bacteria were impractical. The only tests applicable were those based upon serological reactions between bacterial antigens and plague-specific antisera, exemplified by the modified Ascoli test (Larson *et al.* 1951), complement-fixation and haemagglutination tests (Chen *et al.* 1952; Chen & Meyer, 1954), and the fluorescent antibody staining technique.

The fluorescent antibody technique and Larson's modified Ascoli test were used and found to give somewhat similar results except with carcasses and tissues stored for periods up to about 11 months. From Tables 1 and 2 the following comparative results can be noted: of 51 plague-infected mouse carcasses examined by both tests 32 were plague-positive by precipitin tests as contrasted with 42 positive by FA tests. During the first 82 days approximately equal numbers of positives were obtained independently by each test. In addition, approximately equal numbers were positive by one test and negative to the other, or negative by both tests. The difference is found in periods longer than 82 days (i.e. 140 and 340 days at 4°–5° C.) where fluorescent antibody methods yielded positive results in 19 of 21 tests as contrasted with 11 positive in 21 tests by the precipitin technique. It should be pointed out that the results of testing the 340-day specimens by Larson's modified Ascoli methods are somewhat doubtful since many of the extracts were turbid and 2 positive tests were obtained in the tularaemia controls. Unsuccessful attempts were made to eliminate these two false positives and to clarify tissue extracts by centrifugation, the use of highly purified plague-specific antisera, and preabsorption with normal rabbit sera. None of these specimens caused difficulties when examined by fluorescent antibody techniques. It is of interest to note here that the tissues of two guinea-pigs stored for seven years at

room temperature, although positive by fluorescent antibody tests, were negative by the modified Ascoli test. In these two specimens the precipitin test apparently failed owing to insolubility of the plague antigens.

DISCUSSION

During investigations of reported plague outbreaks among wild rodents, particularly in the western United States, workers frequently arrive at the site after the peak of an epizootic has passed. In some cases recently dead animals may be found. Nevertheless, experience with epidemiological investigations has shown that often it is difficult or impossible to obtain fresh specimens. Carcasses in advanced stages of decomposition or in various states of mummification frequently are found in such areas. In many cases complete or even large portions of carcasses are not recovered, and available materials may consist only of fragments of skin, hair, and dry bones. Specimens of this type usually are too old and desiccated for bacterial culture or animal inoculation, and insufficient or unsuitable material is available for extraction and detection of plague antigen by serological tests. Extracts of desiccated, putrefied material may be turbid and react non-specifically, or the plague antigen may be insoluble, as in the case of the 7-year-old guinea-pig tissues (see above).

The results of experimental fluorescent antibody tests reported here indicate the potential utility of this technique in processing field materials in which the presence of viable plague organisms is doubtful. The accuracy of the test was equal to that of Larson's modified Ascoli test in infected tissues up to 3 months of age. Beyond that period the fluorescent antibody test had greater efficacy. The advantages of speed, accuracy, and success in processing small amounts of suspect material, when combined with serologic specificity, suggest that the fluorescent antibody test may be the method of choice in processing tissues from animal carcasses found during field investigations of suspected plague outbreaks.

SUMMARY

Fluorescent antibody techniques were used for detection of plague antigens in infected mouse carcasses stored at 4°–5° C., 20°–25° C. (room temperature) and 37° C. for periods as long as 340 days in certain cases. Tissues of two guinea-pigs infected with *Pasteurella pestis* were examined after 7 years storage at room temperature. The efficacy of the fluorescent antibody test was compared with blood agar plate culture and animal inoculation during periods in which viable plague bacilli were presumed to be present, and compared with Larson's modified Ascoli test for the presence of soluble plague antigens during periods in which plague bacteria were presumed to be non-viable.

Fluorescent antibody tests were superior to animal inoculation or cultural methods in detection of *P. pestis* antigens in mouse carcasses stored for 4 days or longer at 37° C., for 8 days or longer at 20°–25° C. (room temperature), and for 34 days or longer at 4°–5° C. Upon examination of specimens presumed to lack viable plague organisms the fluorescent antibody test and precipitin tests

were approximately equal in efficiency for detection of plague antigens in carcasses stored for 16–82 days at 37° C. and for 16–82 days at 20°–25° C. Ten of 21 mice stored for longer periods (140 and 340 days) at 4°–5° C. were negative by precipitin tests, whereas 19 of the same 21 rodents were positive by fluorescent antibody tests. In addition, after 7 years storage at room temperature tissues of two plague-infected guinea-pigs were negative by Larson's modified Ascoli test but positive by the fluorescent antibody test.

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EXPLANATION OF PLATE 1

Fig. A. Fluorescent antibody stain of plague bacilli in an impression smear of mouse spleen on the day of death.

Fig. B. Fluorescent antibody stain of plague bacilli in an impression smear of spleen from a mouse carcass held 64 days at from 4°–5° C. Note the moderate loss in brilliance of staining and partial lysis of the bacilli.

Fig. C. Fluorescent antibody stain of the putrefied abdominal contents of a mouse carcass stored for 4 days at room temperature. Note the reticular appearance of the slime, which contains brilliantly stained antigenic debris from lysed plague bacilli.

Fig. D. Fluorescent antibody stain of mixed spleen and liver tissues from a plague-infected guinea-pig. The tissues were removed 7 years previously and stored at room temperature (see text). Note the few brilliantly stained particles and the large diffuse mass of stained plague antigen.
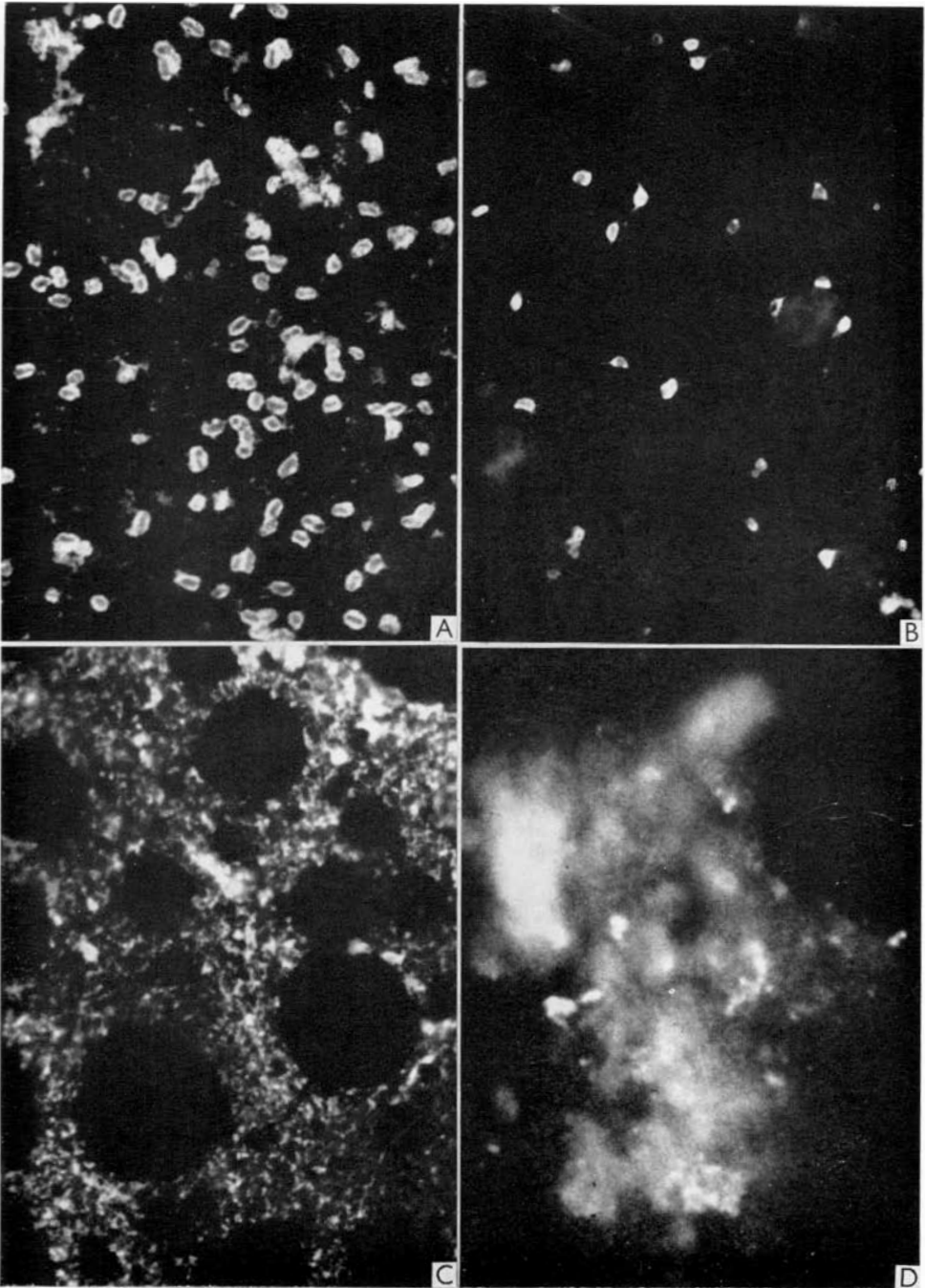
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