Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1966, **34**, 709-714

Pasteurella pestis Detection in Fleas by Fluorescent Antibody Staining*

BRUCE W. HUDSON,[1] LEO KARTMAN[2] & FRANK M. PRINCE[3]

In an effort to develop a method for the rapid field identification of plague-infected fleas, the authors have studied the feasibility of direct fluorescent antibody staining of the midgut contents of fleas fed on mice infected with Pasteurella pestis. *Fluorescent antibodies prepared from antisera derived from rabbits inoculated with the water-soluble* P. pestis *fraction 1b antigen, the somatic antigen of heat-killed* P. pestis *(Bryans strain), and live avirulent (strain A1122) or virulent (Yreka strain) plague vaccines were used in this study.*

This direct staining method proved to be impracticable, but encouraging results were obtained by fluorescent antibody staining of broth cultures of macerates of infected fleas after 24-48 hours' incubation.

The broth enrichment technique has not yet been evaluated in the field, but it is expected to be of value since it is relatively simple to perform and requires only material that can easily be transported to remote areas.

Fluorescent antibody staining techniques for the rapid identification of *Pasteurella pestis* in animal tissues and in bacterial cultures have been developed (Moody & Winter, 1959; Winter & Moody, 1959a, 1959b). The results obtained are specific. Occasionally *P. pestis* strains are found to be deficient in antigenic structure and *P. pseudotuberculosis* strains containing antigenic constituents that produce cross-reactivity with *P. pestis* fraction 1 antigen have caused difficulties (Winter & Moody, 1959b; Quan et al., 1965). The detection of *P. pestis* infection in fleas at present depends on bacterial culture of individual fleas or upon animal inoculation with triturates of individual or pooled fleas. In field locations where there are no facilities for holding large numbers of animals or for bacterial culture and identification of *P. pestis* isolates it is difficult to obtain adequate information pertaining to infections in fleas. Attempts to adapt fluorescent antibody techniques to studies of plague in nature have necessarily involved a study of their feasibility for the examination of infected fleas. Preliminary studies indicated that direct staining of infected flea tissues was not practicable. It was possible, however, to obtain reliable results by using a simplified broth enrichment culture technique.

MATERIALS AND METHODS

Preparation of fluorescent antibody reagents

Antisera. Antiserum to water-soluble *P. pestis* fraction 1b antigen was prepared by intravenous injection of crystalline fraction 1b antigen[4] using three adult New Zealand rabbits. The animals were inoculated with 1 mg of fraction 1b twice weekly for a period of four weeks and were exsanguinated on the tenth day following the last inoculation.

Antisera to avirulent *P. pestis* strain A1122 and virulent strain Yreka were prepared by intravenous inoculation of six rabbits with 6×10^8 living avirulent *P. pestis* of strain A1122 twice weekly for a period of three weeks. After 21 days the rabbits were separated

* From the US Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Technology Branch, Disease Ecology Section, San Francisco, Calif., USA.

[1] Research Biochemist, Biology/Chemistry Section, Technology Branch, Communicable Disease Center, Savannah, Ga., USA.

[2] Scientist Director, US Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Technology Branch, Disease Ecology Section, San Francisco, Calif., USA.

[3] Sanitarian Director and Chief, San Francisco Field Station, US Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Technology Branch, San Francisco, Calif., USA.

[4] From Cutter Laboratories, Berkeley, Calif., USA. Mention of sources of supply is for identification only and does not constitute endorsement by the Public Health Service or by the US Department of Health, Education, and Welfare.

into two groups. One group of three rabbits received an additional course of inoculations of avirulent *P. pestis* strain A1122; the other group received intravenous inoculations of 5×10^7 virulent *P. pestis* strain Yreka twice weekly. This procedure was continued for an additional three weeks. All animals were bled out 10 days after the last inoculation.

Antiserum to *P. pestis* somatic antigen was prepared with Bryans strain (M23) *P. pestis*. The bacilli were grown on peptone-agar slants at 20°C-25°C, harvested in physiological saline, and heated to 60°C for one hour. They were then washed by centrifugation in six changes of physiological saline and resuspended in physiological saline containing 0.5% phenol. Microscopic examination of the suspension yielded counts of 5×10^9 to 8×10^9 dead bacilli per ml. One millilitre of the suspension was inoculated into rabbits twice weekly for a period of six weeks. The six animals inoculated in this fashion were bled 10 days following the final inoculation. Sera from the two animals having the highest titre for washed *P. pestis* of the Bryans strain were then pooled for further use.

Globulin fractions of all *P. pestis* antisera and pooled normal rabbit sera were separated by ammonium sulfate fractionation and were labelled with fluorescein isothiocyanate using techniques described elsewhere (Winter & Moody, 1959a).

Data pertaining to bacterial agglutination titres and fluorescent antibody staining titres are given in Table 1. Fluorescent antibodies to *P. pestis* fraction 1b or live *P. pestis* of strains A1122 and Yreka were fairly specific for fully enveloped organisms cultured at 37°C (these were of the Alexander strain). Antisera prepared with the somatic antigen gave better agglutination and staining of the fraction-1-deficient Bryans strain than of the Alexander strain.

Fluorescent antibody staining. Materials to be examined were smeared on to thoroughly cleaned microscope slides, lightly heat-fixed and stained for 30 minutes. Goldman's (1957) one-step inhibition technique was used as a control for staining specificity. Before staining, all fluorescent antisera were adsorbed with a mixture of acetone powders of beef liver and ground fleas (*Xenopsylla cheopis*) using the Celite column technique previously described (Hudson, 1961). The equipment used and methods of slide examination have also been described (Hudson et al., 1962).

RESULTS

Direct staining of flea midgut smears

Laboratory-reared, unfed, male *Xenopsylla cheopis* were fed on moribund mice infected with *P. pestis*, Alexander strain. A total of 55 fleas were fed on mice with terminal plague septicaemia, placed in individual test-tubes, and held at room temperature (range 20°C-25°C). An additional 25 fleas were fed on normal mice and held under the same conditions. All fleas were subsequently maintained by daily feedings on individual normal white mice. Samples consisting of nine or ten infected and three control

TAB

PASTEURELLA PESTIS AGGLUTINATION TITRES AND FLUORESCENT ANTIBODY STAINING TITRES OF THE FLUORESCEIN ISOTHIOCYANATE-LABELLED GLOBULIN PREPARATIONS USED

Antiserum	Antigen inoculated	Bacterial agglutination titre		Fluorescent antibody staining titre [a]		
		Bryans [b]	Alexander [b]	Bryans, 20°C [c]	Alexander, 20°C [c]	Alexander, 37°C [d]
Fraction 1b	Water-soluble *P. pestis* fraction 1b	None	1 : 180	No staining	No staining	1 : 360 (+3)
Somatic	Washed, heat-killed *P. pestis*, Bryans strain	1 : 80	1 : 10	1 : 40	1 : 20	1 : 1
A1122	Live avirulent *P. pestis*, strain A1122	1 : 4	1 : 360	1 : 10	1 : 10	1 : 360
Yreka	Live *P. pestis*, strain A1122, followed by live virulent *P. pestis*, strain Yreka	1 : 8	1 : 360	1 : 10	1 : 5	1 : 720

[a] Maximum dilution of 1 % labelled globulin solution which yielded moderate to bright staining of organisms.
[b] Prepared according to Baltazard et al. (1956).
[c] Cultured on blood-agar plates 20°C for 48 hours.
[d] Cultured on blood-agar plates 37°C for 48 hours.

fleas were taken at six hours and at two, five, and eight days after the original blood meal. The midgut of each flea was dissected out and macerated in a drop of saline by means of a glass rod. Smears from the midguts were made on microscope slides for fluorescent antibody examination, and on blood-agar plates for bacterial culture. The results of bacteriological and fluorescent antibody examination are shown in Table 2. In general, a large amount of lysed *P. pestis* antigen was noticeable after six hours. The appearance of this lysed antigen during the first day after the infectious blood meal was very similar to that illustrated in a previous report dealing with the detection of *P. pestis* in animal carcasses (Hudson et al., 1962, Plate 1, Fig. C). Very few whole bacteria were noted in smears made of flea midguts six hours after the infectious meal. Nevertheless, at six hours three of the four fluorescent antibodies used gave positive identifications of *P. pestis* in each of the 10 fleas examined. Results of fluorescent antibody examination of fleas from the second through the eighth days yielded poor results when compared with the results of bacteriological culture. Antiserum to *P. pestis* somatic antigen gave positive results in only one case; this was a flea examined five days after the infectious blood meal. The fluorescence observed was very faint and would have been useless for diagnostic purposes. Six additional fleas were examined by use of the antiserum to *P. pestis* somatic antigen ten days after the infectious blood meal. The stomachs of all six fleas were blocked or partially blocked by large *P. pestis* masses in the proventriculus. The masses and stomach contents were dissected out separately for examination. All six fleas were positive by blood-agar plate culture, but the same *P. pestis* organisms failed to stain by the technique used.

Following this initial experiment a series of three essentially similar experiments were performed with male *X. cheopis* and three fluorescent antibody reagents—fraction 1b, strain A1122, and strain Yreka conjugates. Results in all cases were similar. A flea yielding positive results with one conjugate also yielded positive results with the remaining two conjugates. Thus, the data shown in Table 3 are representative and are confined solely to the results of staining with the fraction 1b conjugate. A total of 153 fleas were examined, and of these only 59 gave definite positive results when stained by fluorescent antibody. At the same time a total of 117 fleas yielded positive results when tested by bacteriological culture on agar plates. This latter value would no doubt have been even larger if sterile procedures had been used in processing the fleas, since a large number of the negative bacteriological results were obtained on plates overgrown by contaminating organisms. In addition it is readily apparent that the majority of positive results were

TABLE 2

RESULTS OF DIRECT *PASTEURELLA PESTIS* FLUORESCENT ANTIBODY STAINING OF FLEA MIDGUT SMEARS OBTAINED AT VARIOUS INTERVALS AFTER AN INFECTIOUS BLOOD MEAL

Time after blood meal	Total number of *X. cheopis* tested	Number positive by bacteriological culture	Results of fluorescent antibody examination by various fluorescent conjugates (No. positive/No. tested)			
			Fraction 1	A1122	Yreka	Somatic antibody
Fleas fed on infected mice						
6 hours	10	10	10/10	10/10	10/10	0/10
2 days	9	4	1/9	2/9	2/9	0/9
5 days	10	7	3/10	3/10	3/10	1/10 [a]
8 days	10	3	0/10	0/10	0/10	0/10
10 days	6	6				0/6
Control fleas						
6 hours	3	0	0/3	0/3	0/3	0/3
2 days	3	0	0/3	0/3	0/3	0/3
5 days	3	0	0/3	0/3	0/3	0/3
8 days	3	0	0/3	0/3	0/3	0/3

[a] Staining very dim.

TABLE 3

SUMMARY OF RESULTS OBTAINED BY DIRECT FLUORESCENT ANTIBODY STAINING WITH *PASTEURELLA PESTIS* FRACTION 1b OF MIDGUT SMEARS OF FLEAS AT VARIOUS PERIODS FOLLOWING INFECTIOUS BLOOD MEALS

Day	Total number of *X. cheopis* tested	Condition of flea [a]	Number fed or unfed	Results of fluorescent antibody (FA) [b] test and bacteriological (Bact.) culture					
				FA + Bact. +	FA + Bact. —	FA ± Bact. +	FA ± Bact. —	FA — Bact.+	FA — Bact. —
0 (1-4 hours)	31	A	31	31					
1	18	A	17	6	1 [c]			9	1
		B	1	1					
2	27	A	22	7	2 [c]	2	1	4	6
		B	5	3				2	
3	18	A	12	5			2 [c]	2	3
		B	6	1				5	
4	18	A	13					5	8
		B	5					4	1
5	10	A	4			1			3
		B	6	2				4	
6	14	A	1						1
		B	9					9	
		C	4					4	
8	10	A	5						5
		B	4					2	2
		C	1					1	
10	7	B	3					3	
		C	4					4	
Total	153		153	56	3	3	3	58	30

[a] Condition of flea after blood meal:
A = stomach filled with blood, no *P. pestis* masses.
B = stomach filled with blood, medium to large masses.
C = stomach empty, large masses obstructing proventriculus.

[b] FA results expressed as:
+, staining brilliant, 3+ to 4+; ±, staining dim, 1+ to 2+, of questionable diagnostic value; —, staining not discernible.

[c] Cultures negative owing to overgrowth by contaminants.

obtained during the first few days; no positives were recorded after the fifth day even though the majority of fleas examined were heavily infected, as evidenced by the presence of ventricular masses of *P. pestis*.

It was again noted that a large proportion of the plague organisms appeared to be lysed during the first few hours after the infectious blood meal. Although the numbers of plague organisms present in each flea increased thereafter, the brilliance of the fluorescent antibody staining of each organism decreased until few organisms were stained brilliantly enough for diagnostic purposes after the third or fourth day. Thus, it became apparent that staining of *P. pestis* in fleas with any of the usually available fluorescent antibodies used in work with *P. pestis* was not practical for diagnostic purposes. The failure of *P. pestis* specific fluorescent antibodies to stain *P. pestis* in fleas after a few days was not surprising, since Cavanaugh & Randall (1959), using indirect fluorescent antibody methods, have reported that *P. pestis* organisms in fleas yield only very dim fluorescence with antibodies specific to *P. pestis* fraction 1b antigen or to plague toxin. The reasons for this phenomenon undoubtedly are based upon antigenic changes that occur in the plague organism while it is in the flea. The loss of envelope substance (fraction 1) is known to occur (Cavanaugh & Randall, 1959).

TABLE 4

RESULTS OF *PASTEURELLA PESTIS* FRACTION 1b FLUORESCENT ANTIBODY EXAMINATION OF BEEF-HEART INFUSION BROTH CULTURES OF WHOLE FLEAS

Day after infectious meal	Number of *X. cheopis* tested	Results of tests (No. positive) at indicated intervals following inoculation of broth			
		Fluorescent antibody tests at:			Blood-agar plate culture at:
		24 hours	48 hours	24 or 48 hours	0, 24 or 48 hours
0	6	3	5	6	6
1	7	3	6	7	7
3	6	4	5	5	6
5	6	2	5	5	3
10	7	3	5	5	5
Total	32	15	26	28	27

Staining with fluorescent antisera to *P. pestis* fraction 1 and to live avirulent or virulent *P. pestis* strains should be similar, since Winter & Moody (1959b) have reported that staining with antibody to whole-cell *P. pestis* antigens is virtually indistinguishable from staining achieved with antibody to *P. pestis* fraction 1. Although the antiserum to *P. pestis* somatic antigen stained *P. pestis* cultured at room temperature, it was of little use for staining *P. pestis* organisms in fleas. The reasons for this are not known, but it is possible that residual envelope proteins may have shielded the organisms from the reaction of antibodies with somatic materials. A phenomenon similar to this was reported by Winter & Moody (1959b), who noted that plague cultures containing fully enveloped *P. pestis* cells could be stained only by fluorescent antibodies to somatic antigens after heat destruction of the envelope protein. This was done by heating suspensions of the plague bacillus before making smears for fluorescent antibody examination. Since such methods would not be applicable to materials derived from the midguts of fleas an alternate method was required.

Broth enrichment technique

It was decided that a broth enrichment culture technique might allow the rapid development of envelope antigens in *P. pestis* if cultures were kept at 37°C. Although 28°C is the optimum growth temperature for *P. pestis*, the optimum temperature for production of fraction 1b (37°C) was used since sufficient organisms for microscopic examination were presumed to be present in the blocked stomach of the flea. The following experiment was conducted to test the feasibility of such a technique. Fifty male *X. cheopis* were infected as previously described, and an additional 25 control fleas also were fed at the same time. Fleas were held in groups of 25 in gauze-covered pillboxes and fed on normal white mice every other day. Six or seven infected fleas and three controls were removed at 0, 1, 3, 5, and 10 days following the original blood meal. Each flea was macerated in two drops of beef-heart infusion broth and placed in the bottom of individually numbered Kahn tubes. One loopful of each broth culture was then used immediately for inoculation of a blood-agar plate. The tubes were stoppered with non-absorbent cotton-wool and placed in a humid chamber held at 37°C and 100% relative humidity. Each broth culture was checked by fluorescent antibody staining and blood-agar plate culture at 24 and 48 hours following the original inoculation. The tubes were opened at each time interval, and one loopful of broth was removed from each tube for the preparation of fluorescent antibody smears and the inoculation of blood-agar plates. Fluorescent antibody to *P. pestis* fraction 1b antigen was used for all staining.

Table 4 presents the results obtained by this method. A total of 32 fleas were tested, and fluorescent antibody tests were positive in 28. Twenty-seven of these were verified by bacteriological culture. In general a greater number of fluorescent antibody positives were obtained after 48 hours than

after 24 hours of broth enrichment culture. In certain cases, however, fluorescent antibody examination yielded negative results at one time and positive results at another time. Therefore, the best results were obtained only by fluorescent antibody examination at both intervals.

DISCUSSION

These tests were performed under ideal laboratory conditions, using heavily infected fleas. The technique could not be expected to detect infection in fleas containing small numbers of plague bacilli. Such a technique could be used, however, under conditions in which rapid results are desired and the necessary facilities for a more meticulous procedure are not available, should it be proved effective in field studies. Methods which yield a maximum of information require careful bacteriological culture accompanied by animal inoculation (Quan et al., 1958). Animal quarters and bacteriological equipment for such tests are usually found only in centrally located laboratories, and such examination entails the dispatch of flea specimens to a central location. This may require a week or more. Processing at the central laboratory and the notification of results to the collector in a field location may require another week or more. These periods could be of crucial importance under emergency conditions. If the sacrifice of a certain amount of information is allowable, and a knowledge of only those fleas which are heavily infected is acceptable, then fluorescent antibody staining, using a broth enrichment culture technique, should be of value since all the necessary equipment and materials required for this technique can be easily transported to remote areas.

RÉSUMÉ

Le diagnostic de l'infection à *Pasteurella pestis* chez la puce se fait actuellement par culture bactérienne des puces ou par inoculation à l'animal d'un broyat d'insectes isolés ou réunis en pools. Il est difficile de réaliser ces opérations sur le terrain où l'on ne peut entretenir un grand nombre d'animaux de laboratoire ni faire les cultures nécessaires à l'isolement et à l'identification du bacille. Les auteurs ont recherché si ce diagnostic pouvait être obtenu par la technique de l'immuno-fluorescence.

Ils ont nourri des mâles de *Xenopsylla cheopis* sur des souris moribondes, infectées avec *P. pestis* souche Alexander et essayé de colorer directement leur contenu intestinal. Les anticorps fluorescents ont été préparés à partir de sérums de lapins inoculés avec la fraction hydrosoluble 1 b de *P. pestis*, l'antigène somatique de *P. pestis*, la souche avirulente A1122 et la souche virulente Yreka. Les résultats obtenus du deuxième au huitième jour après l'infection ont été médiocres.

Les auteurs ont cherché à mettre au point une technique de culture simplifiée sur bouillon. Si la température optimale pour la croissance du bacille est 28°C, la température optimale pour la production de la fraction 1 b est 37°C. Cinquante mâles de *X. cheopsis* ont été infectés comme il a déjà été décrit, en même temps que 25 témoins. Réparties en groupes de 25, les puces ont été nourries un jour sur deux sur souris blanches normales. Six ou sept puces infectées et trois témoins ont été prélevées 0, 1, 3, 5 et 10 jours après le repas infectant et mises à macérer dans deux gouttes de milieu à l'infusion de cœur de bœuf, qui a servi à ensemencer immédiatement des boîtes de gélose au sang pour vérifier bactériologiquement la culture. Le même bouillon a servi à la recherche de l'immuno-fluorescence avec des anticorps anti-fraction 1 b: sur 32 puces examinées par cette épreuve, 28 ont donné une réaction positive, vérifiée pour 27 d'entre elles par culture.

REFERENCES

Baltazard, M., Davis, D. H. S., Devignat, R., Girard, G., Gohair, M. A., Kartman, L., Meyer, K. F., Parker, M. T., Pollitzer, R., Prince, F. M., Quan, S. F. & Wagle, P. (1956) *Bull. Wld Hlth Org.*, **14**, 457-509

Cavanaugh, D. C. & Randall, R. (1959) *J. Immunol.*, **83**, 348-363

Goldman, M. (1957) *J. exp. Med.*, **105**, 557-573

Hudson, B. W. (1961) *Bull. Wld Hlth Org.*, **24**, 291-292

Hudson, B. W., Quan, S. F. & Kartman, L. (1962) *J. Hyg. (Lond.)*, **60**, 443-450

Moody, M. D. & Winter, C. C. (1959) *J. infect. Dis.*, **104**, 288-294

Quan, S. F., Fintel, H. von & McManus, A. G. (1958) *Amer. J. trop. Med. Hyg.*, **7**, 411-415

Quan, S. F., Knapp, W., Goldenberg, M. I., Hudson, B. W., Lawton, W. D., Chen, T. H. & Kartman, L. (1965) *Amer. J. trop. Med. Hyg.*, **14**, 424-432

Winter, C. C. & Moody, M. D. (1959a) *J. infect. Dis.*, **104**, 274-280

Winter, C. C. & Moody, M. D. (1959b) *J. infect. Dis.*, **104**, 281-287