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Source: Bulletin of the Wildlife Disease Association, 3(2) : 50-59

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-3.2.50

The Use of the Passive Hemagglutination Test in Epidemiologic Investigations of Sylvatic Plague in the United States

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Received for publication 10 February, 1966

ABSTRACT

Serologic epidemiology was applied to investigations of wild rodent plague in areas of California, Arizona, Colorado, and Utah. Results indicated that the passive hemagglutination test can be of value in detecting antibodies to the water-soluble Fraction Ib antigen of *Pasteurella pestis* in wild rodent or other mammalian sera. Positive tests were obtained with sera of mammals from all areas with a concurrent or recent history of wild rodent plague. Thus, the current status of *P. pestis* infection in mammals was determined, as well as a retrospective determination of past infection. Some evidence was found suggesting that certain plague-resistant rodents that have a wide geographic distribution (i.e., *Peromyscus maniculatus*) may serve as "indicator animals" in plague investigations.

INTRODUCTION

Investigations of the immune status of host populations have become established as being of significant importance in epidemiologic studies of wild rodent plague. A number of investigators have recommended the preferential use of the passive hemagglutination test as adapted to plague diagnosis by Chen and Meyer[1] and Neel and Baltazard[2]. This test was found to be more sensitive and easier to perform than bacterial agglutination or direct conglutination and was recommended for use in wild rodent serology[3].

Recent use of this highly sensitive test in investigations of epizootic plague in South Africa[4], the Soviet Union[5] and the United States[6,7] has emphasized its importance for the retrospective diagnosis of *P. pestis* infection of wild rodents. Workers have in general concluded that the passive hemagglutination reaction should be used in investigations of epizootics of plague and that it can aid in the characterization of a natural focus by defining the zones of transmission, the plague-free zones, and thus the enzootic area.

Perhaps the first report of an intensive serologic study of a plague pocket in the United States was communicated by our laboratory[8]. During successive plague epizootics in 1961-1962 and 1962-1963, from 60 to 80 per cent of sera from the California vole, *Microtus californicus* gave positive results when tested by the passive hemagglutination test[8]. In a more recent study of the same area the presence of serum antibody in 90 to 100 per cent of animals trapped at separate sites within the plague pocket was not at all unusual[9].

The serologic technique has been used by our laboratory during epidemiologic investigations conducted at the request of a number of State and Federal health

[1] Dr. Hudson is Research Biochemist and Dr. Kartman is Scientist Director.

authorities in several Western States. These studies included investigations made subsequent to reports of human plague cases, investigations of reported epizootics among wild rodents, and surveillance of areas in which epizootics of wild rodent plague had occurred in the past. Therefore they represent several of the many types of wild rodent plague situations in the United States which the public health worker might be called upon to investigate. This communication presents the results of these serologic studies.

METHODS AND MATERIALS

Bacteriology

Since standard techniques for surveillance of wild rodent plague have been thoroughly discussed by a number of authors[10], the details of bacteriologic methods or findings are not presented in the following sections. In general, animals were trapped, bled, and their fleas and tissues were processed by inoculation of white mice, guinea pigs and/or blood agar plates. In areas in which die-offs of small mammals had occurred, frequent use was made of fleas collected by swabbing of rodent burrows with white outing flannel. Mummified or putrefied animal carcasses were processed by flourescent antibody techniques[11].

Blood Sampling Techniques and processing of animals and fleas

All small mammals were placed in 1-quart mason jars and taken to a centralized work area. There they were anesthetized with ether, and blood samples were taken by heart puncture. Very small rodents were bled using a disposable 26-27 gauge needle and 1-ml tuberculin syringe. Larger animals were bled using a standard 2 or 5 ml disposable syringe. Blood was expressed from the syringe into a sterile culture tube lying horizontally. After the blood had clotted for 30 minutes to 1 hour, the culture tubes were placed upright in a standard test tube rack and were held in a refrigerator overnight. Serum drained easily from the clots, and could be pipetted from the bottom of the tubes without difficulty. All sera were placed in 6 mm x 50 mm culture tubes, capped by a rubber stopper, and frozen in dry ice for shipment. Frequently, when collecting blood of small rodents the blood was diluted with one volume of phosphate-buffered physiologic saline (PBS)*† in order to increase serum volume for ease of handling. The sera resulting were regarded as a 1:2 dilution with no attempts being made to compensate for cell volume.

After blood samples had been taken, all rodents were killed and, together with their fleas, placed in sealed plastic bags and frozen on dry ice for shipping to the laboratories in San Francisco. There the animals were thawed, fleas were removed, and both tissues and fleas were examined by standard bacteriologic methods[10].

The Passive Hemagglutination Test

Reagents for the test were prepared by the recommended procedures[10] with minor modifications. Fresh sheep blood was collected in Alsevier's solution and held under refrigeration for 2 to 4 days before use. At that time the sheep erythrocytes were washed 3 to 5 times by gentle centrifugation in PBS. A portion of the washed cells was resuspended to 5 per cent v/v in PBS and warmed to 37°C. An equal volume of freshly made 1:10,000 solution of tannic acid in warm (37°C) PBS was then added and the mixture incubated with constant gentle shaking for 15 minutes at 37°C. The cells were then centrifuged gently for 5 minutes and washed once again in PBS. The tanned cells were resuspended to a concentration of 5 per cent in PBS and one volume of a 50 μg/ml (in PBS) solution of water soluble antigenic Fraction IB of *P. pestis* was added. This mixture was held at room temperature with continuous gentle stirring for 30 minutes. The cells were then washed once in PBS and twice with a 1:250 solution of heat inactivated normal rabbit serum (NRS) in PBS. The cells were finally resuspended to 0.1 per cent in the 1:250 dilution of NRS in PBS. All sera to be tested as well as the NRS used as a diluent were inactivated at 60°C for 20 minutes.

The test was performed with the "Microtiter" equipment manufactured by Cooke Engineering Company, Alexandria, Virginia†. This consisted of a series of loops and dropping syringes, calibrated to contain or drop 25-microliter quantities, and transparent plastic plates containing round-bottomed wells in which the test was conducted. The plates contained 8 series of 12 wells each. Three drops of a 5- to 10-per cent suspension of the washed sheep erythrocytes in 1:100 NRS

* FTA Hemagglutination Buffer, Dehydrated. B-D Laboratories, Inc., Baltimore, Maryland.

† Trade names are furnished for purposes of identification only, and do not constitute endorsement by the U. S. Public Health Service or the Department of Health, Education, and Welfare

were added to the first well. To this was admixed one loop of the sera under test. After mixing, the cells were allowed to settle for 2-3 hours. One drop of 1:100 NRS in PBS was then added to each of the remaining wells. A sample of the supernatant liquid in the first well then was taken up in a loop and diluted serially in the remaining wells. One drop of the 0.5-per cent solution of tanned and antigen-coated erythrocytes was then added to each dilution. Commencing with undiluted sera the final dilutions tested were 1:16 through 1:8192.

Controls

All tests were controlled using known negative and positive sera as recommended[10]. It was found advisable to remove a portion of the tanned cells before coating them with *P. pestis* antigen. These cells then were washed with 1:250 NRS and used with each test serum side-by-side with the antigen-coated cells as a control for nonspecific agglutination[12].

The Areas Studied

An historical summary of the plague history of the areas studied is given in table 1. For purposes of the present report, we did not consider it essential to present a detailed epidemologic account of each area. It is sufficient to indicate that each of the areas has had a history of plague in wild rodents and their fleas. In the case of Houck, Arizona and Raymond, Colorado the presence of plague was first heralded by human infections; subsequently, no isolations of *P. pestis* have ever been made from small mammals or their fleas.

SEROLOGICAL RESULTS AND DISCUSSION

The numbers of sera obtained from each area and subsequently tested by the passive hemagglutination technique are shown in table 2. Blood sera from mammalian species belonging to 15 genera were tested. No positive results were obtained from species of *Dipodomys, Eutamias, Neotoma, Ochotona, Mus, Perognathus, Sorex, Sylvilagus,* or *Zapus.* However, in these cases, due to the small number of sera tested, the negative results cannot be considered to be significant. Laboratory experimentation has demonstrated that positive serologic results are likely to be obtained more frequently from species resistant to plague[19]. Results of a 2-year investigation in a plague pocket in San Mateo County, California, agreed with this concept since positive results were obtained only in resistant *Microtus californicus, Peromyscus maniculatus* and, as demonstrated in this report, in moderately resistant *Rattus norvegicus.* The susceptible species checked during these studies, *Mus musculus* and *Reithrodontomys megalotis,* although tested in fairly small numbers, yielded negative results. This led us to believe that a similar situation should prevail elsewhere. However, 5 of 9 *Reithrodontomys megalotis* sera collected from Indian Farm Canyon in Utah (Area C, table 3) yielded positive results with titers ranging from 1:32 to 1:256. This conflicting result may be due to differences in host strains (i.e., a plague-resistant local strain of harvest mice), to differences in flea species and their efficiency as vectors, or it may be

Table 1. *Summary of plague history of the areas studied.*

Location	Date	Isolation of *Pasteurella pestis* from Dom. Rats	Wild Rodents	Flea Pools	Ref.
Southern Marin County, Calif.	Aug.-Oct., 1942	—	—	+	13
Lava Beds Nat. Monu., Siskiyou Co., Calif.	Jan.-Feb., 1962	—	+	+	13
Park County, Colorado	1945 - 1947	—	+	+	14
Chubbs Park, Park Co., Colorado	Summer, 1959	—	+	+	15
Near Fairplay, Park County, Colorado	May-Aug., 1963	—	+	+	13
Arapahoe County, Colorado	July, 1963	—	+	+	13
Indian Farm Canyon, Juab County, Utah	March, 1961	—	+	+	16
Houck, Apache County, Arizona	Dec., 1963	—	*	—	13
Raymond, Boulder County, Colorado	Sept., 1957	—	*	—	13
San Bruno Mt., San Mateo County, Calif.	1954 - 1955	+	+	+	17, 18

* Human plague cases, no evidence from animals at the time.

Table 2. *Mammal sera collected from different areas in the Western United States.*

Mammal Species	Number Collected And Tested Collection Areas[1] A	B	C	D	E	F	G	H	Total No. Tested	Total No. Positive	% Positive
Citellus beecheyi					1				1	0	—
C. lateralis					11			11	22	0	—
C. richardsoni	4								4	0	—
C. tridecemlineatus	12	24							36	8	22
Dipodomys heermani			3		13				16	0	—
D. ordii		4							4	0	—
Eutamias amoenus					52				52	0	—
E. dorsalis			10						10	0	—
E. minimus								21	21	0	—
Microtus californicus				22		202			224	8	3.5
M. montanus			17		3			63	83	4	4.8
Mus musculus				8					8	0	—
Neotoma albigula							5		5	0	—
N. cinerea	1				17				18	0	—
N. leucopus			1						1	0	—
N. mexicana							1	2	3	0	—
Ochotona princeps					1				1	0	—
Onychomys leucogaster		11					4		15	1	6.7
Perognathus hispidus	1	10							11	0	—
P. parvus					131				131	0	—
Peromyscus crinitus			3		32				35	7	20
P. leucopus							12		12	0	—
P. maniculatus	94	36	127	25	329	245	46	22	924	74	8.0
P. nasutus							40		40	1	2.5
P. truei			29		9		5		43	1	2.3
Sorex spp.					2	5			7	0	—
Sylvilagus spp.	4	1					1	2	8	0	—
Rattus norvegicus				57					57	7	12
Reithrodontomys megalotis			9	1	4	13	8		35	5	14
Zapus spp.								7	7	0	—

[1] A. 15 miles East of Fairplay, Park Co., Colorado; July-Aug., 1963. B. 20 miles East of Denver, Arapahoe Co., Colorado; July, 1963. C. Indian Farm Canyon, Juab Co., Utah (Sera supplied through the courtesy of Dr. B. D. Thorpe, University of Utah); March, 1961 to Nov., 1962. D. San Bruno Mountain, San Mateo County, California; Nov., 1963 to May, 1964. E. Lava Beds National Monument, Siskiyou Co., California; June and Sept., 1962; Jan. - Feb., 1963. F. Southern Marin Co., California; Feb. to May, 1964. G. Houck, Apache Co., Arizona; Jan., 1964. H. Raymond, Boulder Co., Colorado; July - Aug., 1964.

indirect evidence for the existence of *P. pestis* strains of reduced virulence. The latter hypothesis may be correct because of reports of isolations of strains of *P. pestis* of low virulence from this area[20].

Positive serologic results were obtained in the genera *Citellus, Microtus, Onychomys, Peromyscus, Rattus* and *Reithrodontomys*. A detailed breakdown of these data is shown in table 3 which lists results by species, area, and known plague history. Inspection of table 3 shows that positive serologic results were obtained in all cases in which *P. pestis* isolations were made concurrently with serum collections. Only two areas gave negative serologic results. In one case, the last known occurrence of wild rodent plague was in 1942, twenty-two years before the present survey was conducted. In the second case, the sole evidence for the presence of wild rodent plague was the circumstances surrounding a human case of plague which occurred in 1957, seven years before the serum survey was conducted. In the remaining positive areas, serologic evidence of *P. pestis* infection in wild rodents was present concurrently with bacteriologic isolations of *P. pestis*. In Area E, the Lava Beds National

Table 3. *Rodent species possessing serum antibodies to* Pasteurella pestis *Fraction Ib antigen.*

	Bacteriologic isolations of *Pasteurella pestis* Concurrent with serum collection							
Area[1]	A		B		C		D	
Species	Number pos./tested	GMT[2]	Number pos./tested	GMT	Number pos./tested	GMT	Number pos./tested	GMT
Citellus tridecemlineatus	8/12	110	0/24					
Microtus californicus							8/22	230
M. montanus					4/17	40		
Onychomys leucogaster			1/11	2048				
Peromyscus crinitus					0/3			
P. maniculatus	12/94	200	3/36	60	21/127	50	8/25	140
P. nasutus								
P. truei					1/29	128		
Rattus norvegicus							7/57	390
Reithrodontomys megalotis					5/9	80	0/1	

[1] Areas A through H are identifed in Table 2, footnote.
[2] GMT - geometric mean titer (Reciprocal)

Table 3 Continued

Bacteriologic isolations of *Pasteurella pestis*							
Prior to serum collection				Not isolated - prior human case			
4-12 months		22 years		4 months		7 years	
E		F		G		H	
Number pos./tested	GMT	Number pos./tested	GMT	Number pos./tested	GMT	Number pos./tested	GMT
		0/202					
0/3						0/63	
				0/4			
7/32	70						
27/329	120	0/245		3/46	510	0/22	
				1/40	512		
0/9				0/5			
0/4		0/13		0/8		0/7	

Monument, serologic positives were obtained from deer mice as long as a year after the last bacteriologic isolation of the plague organism although several collections of sera during intervening months had given negative results. In Area G, which was surveyed in connection with a human plague case 4 months previous to the collection period, the sole evidence pointing toward the wild rodent source of human infection was the four positive serum samples indicated in table 3. Additional evidence in Area G, not tabulated, consisted of positive results obtained from dogs associated with the human case. Three of the 4 dogs which were tested showed positive passive hemagglutination titers ranging from 1:128 to 1:512. Serum samples also were collected from 3 of the 4 dogs captured in the San Bruno Mountain study, Area D. Two of these dogs, one an adult female and the other a juvenile male, yielded positive passive hemagglutination titers of 1:256 and 1:128, respectively.

These results may indicate that indirect evidence of infection in wild rodents and other mammals can be obtained by serologic tests of predator and carnivore sera. However, in view of the lack of experimental information pertaining to this type of test in dogs, the results should be regarded with some caution since there are reports of precipitins in the normal sera of numerous species which react with *P. pestis* antigen[21].

In addition to the possible presence of "natural" antibodies it should be emphasized that serologic tests for antibodies to *P. pestis* Fraction I antigen are not absolutely specific and may cross-react with the antigens of certain *P. pseudotuberculosis* strains[22]. Thus, with this particular problem, in the absence of recent or concurrent bacteriologic evidence of plague infection in the specific animal and in the area being studied, serologic positives *per se* should not be taken as unequivocal evidence of infection. These reservations are probably of more significance when applied to serologic examination of predator species, in view of the paucity of information pertaining to such animals. Current work in Africa has emphasized this matter. In his studies of the Kenya plague focus, Dr. R. B. Heisch states, "I suggest that most of the high antibody titres to *P. pestis* in Kenya rodents are due to *P. pseudotuberculosis*..."‡

A noteworthy feature of the data in table 3 lies in the consistency in which serum positives were found in *Peromyscus maniculatus*. This wild rodent species was found in all the areas studied and is the most widespread small rodent in North America. Because of the wide distribution of the deer mouse and the relative stability of the serologic results obtained, we feel that major efforts should be made to secure serum samples from this species in epidemiologic studies of wild rodent plague conducted in the Western United States. On theoretical grounds it appears that this species could be used as an "indicator animal" in such investigations. This does not mean to imply that *Peromyscus maniculatus* might be the sole species involved in a plague outbreak. On the contrary, the data implicate additional species in each of the areas studied.

The demonstration that diagnosis of *P. pestis* infection by serologic methods is possible in Norway rat populations (Area D, table 3), as well as the relative ease and economy with which such methods can be applied should be of interest to workers concerned with maintaining plague surveillance in urban or feral rat populations. Although the present investigation was conducted during or immediately following a plague epizootic, under conditions conducive to the recovery of *P. pestis* from both animal tissues and fleas, serologic methods also have a potential value during interepi-

‡ Personal communication to L. K., 22 June, 1966.

zootic periods when bacteriologic recoveries are exceptionally difficult to obtain.

Workers in Hawaii[7] have made use of serological tests to examine an area where plague was formerly enzootic. They reported finding antibodies, both in mongooses and in susceptible rodents, in both historically well known enzootic areas and in apparently "new" areas. Their study thus emphasizes the need to examine serological results in the light of known background information concerning the susceptibility of the animal species involved, the previous history of a given area with respect to the occurrence of plague, and concurrent isolation of the plague organism from host and vector populations.

During a previous study in the San Francisco Bay area, a definite seasonal fluctuation in numbers of animals possessing serum antibodies to *P. pestis* Fraction I antigen was noted[8]. Figure 1 shows the fluctuations in antibody rates detected in a study area near San Francisco, California. It can be seen that the results of serum collections made solely during the summer months could have led to erroneous conclusions regarding the degree of infection present in small rodents. Theoretically, it can be assumed that a similar phenomenon may have influenced the serologic results from the Lava Beds National Monument (Area E, tables 2 and 3). Sera were collected from rodents at the Lava Beds National Monument over a period of several months. The great majority of the positive sera were from collections in January and February of 1963, the same season in which overt wild rodent plague had been detected in 1962. The two rather extensive collections made at the Lava Beds National Monument during the summer of 1962 yielded equivocal results in that only two *P. maniculatus* of a total of 201 collected possessed serum antibody to *P. pestis*. Had collections been limited to this period alone, it might

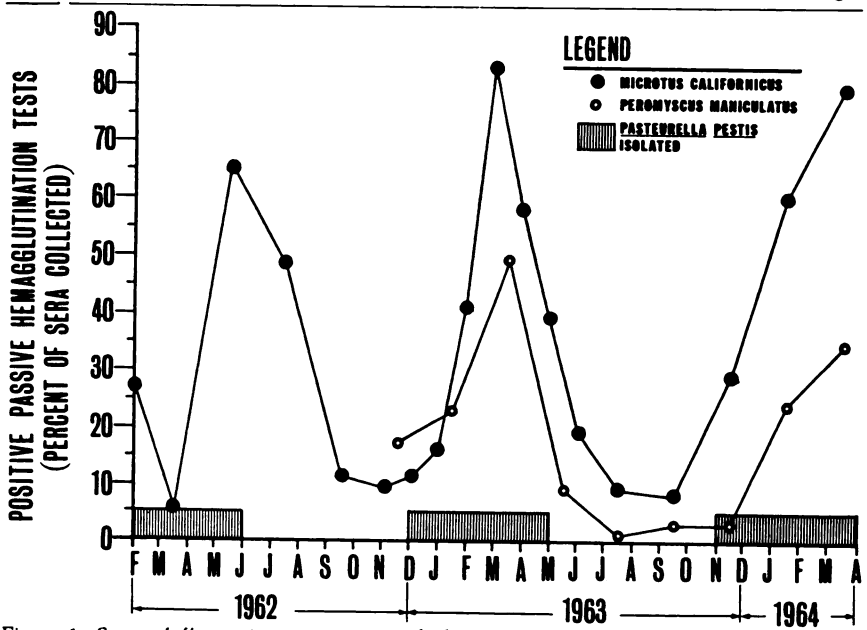

Figure 1. *Seasonal fluctuations in serum antibody rates in the California vole,* Microtus californicus, *and the deer mouse,* Peromyscus maniculatus, *collected in the San Bruno Mountain "plague pocket," San Mateo County, California. (Area D, see table 2).*

have been concluded that wild rodent plague had disappeared from the area. In view of this it is apparent that negative serologic results obtained during one month or season of the year cannot in themselves be used to ascertain whether an area is free from plague. It is also apparent that recent or concurrent infection of wild rodents with *P. pestis* can be detected efficiently by means of serologic tests, and that such detection is possible even in the absence of bacteriologic evidence of infection.

It is evident from these data that serologic studies of sylvatic plague carry the work beyond the "confirmation" of plague in nature as demonstrated by conventional bacteriologic techniques. With the reservations noted above on the problem of cross-reactivity, serologic methods can be employed independently of other methods to indicate the presence of *P. pestis* in animal populations. They also can provide additional information on the spatial or geographic extent of infection[4,5,9,23], and on the seasonal or other cyclic changes in rates of infection (8; figure 1). It has also been demonstrated that rodent populations having a high order of resistance to clinical plague[18,24] nevertheless are subject to widespread epizootics of infection with *P. pestis*[8,9].

ACKNOWLEDGEMENT

We acknowledge the advice of Dr. T. H. Chen, George Williams Hooper Foundation, University of California, San Francisco Medical Center, and Dr. M. I. Goldenberg, C.D.C., San Francisco Field Station. We also wish to thank Dr. B. D. Thorpe for making available a valuable series of wild rodent sera from the Indian Farm Canyon area in Utah. Mr. T. J. Quan, San Francisco Field Station, gave invaluable laboratory assistance.

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