

*Survival of Plague Organisms in Refrigerated Animal Carcasses*¹

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AT THE present time, sylvatic plague in various species of wild animals is chiefly diagnosed and studied by inoculation of test animals with pools of fleas and other ectoparasites collected from wild animals. While one cannot question the excellent work that has been done by this method or the immense amount of data that has been assembled, several questions remain unanswered. Instances have been observed in which enzootic plague remained static for a long period of time and then assumed increased proportions with little or no alteration of the flea ratio, or else, the percentage of plague infection was maintained at a time when the flea ratio was at a very low level. Such observations have been made by C. W. Clanton, State of Washington, Department of Health (personal communication) on a number of occasions, and for one particular outbreak the spread was so rapid and extensive that he felt that flea transmission was improbable and therefore suggested the possibility of pneumonic plague among some of the wild rodents.

The technique of studying sylvatic plague only by means of flea collections would not give information on other possible types of plague transmission. These other methods of transmission may also be important. It is, of course, obvious that practical difficulties arise when one attempts to work with animal tissues in the field. Not only is the danger materially increased to the field operator, but in addition it is very unsatisfactory to attempt to maintain laboratory animals under field conditions. If one proceeds by sending tissues to a central laboratory, the problems of preservation and loss by contamination and autolysis become very serious. In answer to the suggestion that whole animal carcasses of the smaller species, such as wild mice, voles, etc., be sent either frozen or under refrigeration, the objection has been raised by some that plague organisms would not survive low temperatures under such conditions or else would be greatly diminished in number.

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The collection of rodents is usually done by means of snap traps or some similar technique, and therefore only animals healthy enough to be active and moving around are obtained. It is obvious that animals in advanced stages of plague would not be active and hence would be missed by trapping techniques. In advanced stages of the disease enormous numbers of plague bacilli are present throughout all of the tissues; but in the earlier stages, or in chronic plague, there may be relatively few organisms present. Therefore, any method for determining the presence of plague infection must be capable of demonstrating the presence of the organisms when only small numbers are present in the tissues. A method which might be suitable when dealing with the large numbers of bacilli present in the acute phase of the disease might be ineffective when dealing with the smaller numbers present in early or chronic stages.

To evaluate the results of our collections and to answer suggestions that plague bacilli are not well preserved in frozen carcasses, we have undertaken a series of experiments to determine how well these organisms survive exposure to low temperatures while still in the original host tissues.

It was felt that the most suitable way to determine the effect of low temperatures on survival of the organism in tissue was to compare end-points of serial decimal dilutions of spleen emulsions from mice immediately after death and from mice that had been held at low temperatures for varying periods of time after dying of plague infection. A single strain of *Pasteurella pestis* was used that had recently been recovered in this laboratory by the injection of fleas from sage brush voles (*Lagurus curtatus*) collected by Clanton.

The original series of white mice (CFW strain) was inoculated subcutaneously with 0.5 ml of a 10^4 dilution of a broth culture of the above strain. The animals died fairly uniformly on the fourth day, and the carcasses were used to initiate these experiments. For tests on freshly dead mice, the animals were autopsied and the entire spleen removed and weighed immediately on a Roller Smith Balance with an accuracy of better than 0.1 mg. After weighing, the spleen was placed in a sterile mortar and pestle and ground to an even homogeneous pulp without the addition of sand or other material. Because of the nature of the organ and particularly because of its usually greatly congested state such grinding was easily done. To increase the uniformity of the procedure, a standard period of five minutes hand grinding was adopted. The ground spleen was diluted with 49 times its weight of sterile peptone solution and well mixed to give a 1 to 50 dilution based on the weight of the original spleen. This was serially diluted in tenfold steps, using a separate sterile pipette for each step. For each dilution two normal white mice were

injected subcutaneously with 0.5 ml each of dilutions equivalent to 1 ml each of dilutions 10^5 through 10^9 . Most of the deaths occurred by the fourth or fifth day, but the survivors were observed for a period of three weeks. On any one day usually two (at the most four) mice dead of plague were titrated in this manner. Mice dying in these experiments were refrigerated and held for varying lengths of time; then titrated and compared with mice which had just succumbed to plague infection.

TABLE 1. TITRATION OF SPLEENS OF FRESH DEAD PLAGUE MICE

Original Mouse No.	Concentration of Spleen Emulsion in Inoculum					
	10^5	10^6	10^7	10^8	10^9	10^{10}
1	2/2*	2/2	2/2	2/2	2/2	
2	2/2	2/2	2/2	2/2	0/2	
3	2/2	2/2	2/2	2/2	1/2	
4	2/2	2/2	2/2	2/2	2/2	
5	2/2	2/2	2/2	2/2	0/2	
6	2/2	2/2	2/2	2/2	2/2	
7	2/2	2/2	2/2	1/2	0/2	
8	2/2	2/2	2/2	2/2	1/2	
9	2/2	2/2	2/2	2/2	1/2	
10	2/2	2/2	2/2	2/2	2/2	
11	—	2/2	2/2	2/2	0/2	0/2
12	—	2/2	2/2	1/2	1/2	0/2
Total	20/20	24/24	24/24	22/24	12/24	0/4

* Numerator indicates number of mice dying of plague; denominator indicates number of mice injected.

Mice that died in this experiment were taken at random and placed at either refrigerator temperature (5°C) or dry-ice temperature (-78°C) for four days. After four days at these temperatures, the carcasses were thawed, if necessary, and the spleens were removed, weighed, ground, and diluted in the same manner as were the spleens of freshly dead mice. In any one experiment, freshly dead mice were titrated and compared with the same number of mice that had been held at low temperature for four days.

The results of these experiments were that titrations of spleen emulsions of 12 mice that had just died of plague infection killed all of the test animals in doses equivalent to 1 ml of 10^5 through 10^7 dilutions. A sharp 50-per-cent death rate was obtained at the 10^9 dilution. Ten mice which had been held at

5°C for four days before titration of spleen emulsion killed 20 out of 20 test mice at 10^5 and 10^6 dilutions, 19 out of 20 at 10^7 , 14 out of 20 at 10^8 and 12 out of 20 at 10^9 (see Table 2). Ten mice held at -78°C on dry ice for four days before titration of spleen emulsions killed the test animals as follows: 20 out of 20 in both the 10^5 and 10^6 dilutions, 19 out of 20 in 10^7 , 17 out of 20 in 10^8 dilutions, and 11 out of 20 in 10^9 dilution (see Table 2).

The period of four days was chosen because it seemed to be the maximum time that mice could be held at 5°C before autolysis proceeded to an extent which rendered autopsy difficult. That is, four days seemed to represent the time that mice could be held under ordinary refrigeration, in the field, and still be suitable for use in plague determination. It is evident from the above results that the 50-per-cent end-point reached by titrations of spleen emulsions of mice dying of plague is a dilution of 10^9 regardless of whether the mice had just died of plague or whether they had been held under refrigeration at 5°C or had been held at the temperature of dry ice, -78°C , for the same length of time.

Under certain conditions of field operations, four days would be too short a time interval to allow for collection and shipping to a central laboratory. However, it might well be possible that sufficient supplies of dry ice could be taken into the field or else access might be had to a deep freeze in which animals could be held for a greater length of time. In view of these possibilities, the survival of plague organisms in tissues of infected animals was studied for the time interval of seven days at the temperature of a standard deep-freeze (-20°C) and also when preserved by dry ice (-78°C). Tests on mice held at 5°C were not carried out for this time interval because autolysis was too advanced.

The technique was the same as that used for the shorter time interval, and serial dilutions of spleen emulsions were compared with those from mice freshly dead of plague. Ten mice, which had been held in a deep freeze for seven days (-20°C) after death from plague, were tested with the following results: 20 out of 20 mice died when inoculated with 10^5 dilution, 19 out of 20 died when inoculated with 10^6 and 10^7 dilutions, 17 out of 20 died when inoculated with 10^8 dilution, and 5 out of 20 died when inoculated with 10^9 dilution. These results would indicate that the 50-per-cent end-point would fall somewhere between the 10^8 and 10^9 dilutions or, expressed another way, there appeared to have been approximately half a decimal dilution drop in the infective dose as compared with freshly dead mice (see Table 2).

Spleen emulsions from ten mice which had been held at -78°C on dry ice for seven days after dying from plague infection were titrated in the same

way and compared with similar spleen emulsions of mice freshly dead of the disease. Spleen emulsions from mice held at this temperature killed 20 out of 20 test animals inoculated with 10^5 dilution, 18 out of 20 inoculated with 10^6 dilution, 12 out of 20 test animals inoculated with 10^7 dilution, 9 out of 20 inoculated with 10^8 dilution, and 2 out of 20 inoculated with 10^9 dilution. This would appear to indicate a drop of approximately one decimal dilution as compared with spleen emulsions from mice freshly dead of plague (see Table 2).

In view of the apparent drop of one decimal dilution in the L.D.₅₀ after the mice had been frozen with dry ice for seven days before spleen titration, the experiment was repeated. On the second test somewhat different results were obtained which indicated that there had not been as great a drop in titer as had been suggested by the first experiment. As shown in Table 2, Group 5b, all of the mice inoculated with doses equivalent to 1 ml of 10^5 and 10^6 dilutions died as did 19 out of 20 inoculated with the equivalent of 10^7 , 16 out of 20 inoculated with 10^8 , and 14 out of 20 inoculated with 10^9 equivalent doses.

The conclusion can be drawn from the above experiment that there would be at most a tenfold reduction in the infective dose from animals that had been frozen, either by dry ice or in a deep freeze, for a period of one week from the time of collection before animal inoculation was carried out. All of the above experiments indicate that it is quite feasible to collect animals,

TABLE 2. TITRATION OF SPLEENS OF MICE DEAD OF PLAGUE AND HELD FOR VARYING PERIODS OF TIME AT DIFFERENT TEMPERATURES

Group	Number of Mice Titrated	Period Held	Temp.	Number of Mice Dying at Each Concentration of Spleen Emulsion				
				10^5	10^6	10^7	10^8	10^9
1	12	Fresh	—	20/20*	24/24	24/24	22/24	12/24
2	10	4 days	5 C	20/20	20/20	19/20	14/20	12/20
3	10	4 days	-78 C	20/20	20/20	19/20	17/20	11/20
4	10	7 days	-20 C	20/20	19/20	19/20	17/20	5/20
5a	10	7 days	-78 C	20/20	18/20	12/20	9/20	2/20
5b	10	7 days	-78 C	20/20	20/20	19/20	12/20	14/20
Total	62			120/120	121/124	112/124	95/124	56/124

* Numerator indicates number of mice dying of plague; denominator indicates number of mice injected.

at least the smaller species, in the field and maintain them in the frozen state for a period of at least a week before they are examined in some central laboratory. Such freezing can be done either by means of dry ice or else by means of a commercial deep-freeze without a reduction of more than one decimal dilution in the number of infective doses. In view of other experiments we have carried out, even this apparent reduction may be due, in part at least, to experimental error.

Our data on the length of time that mice may be maintained at -20°C before there is a significant loss in the number of infective doses are more limited but do suggest that in frozen carcasses the organisms are maintained relatively unaltered for long periods of time. One mouse held at this temperature for 48 days before titration killed 2 out of 2 test animals at 10^5 , 10^6 , and 10^7 dilutions and 1 out of 2 test animals at 10^8 and 10^9 dilutions. Another mouse held at this temperature for 76 days before spleen titration killed 2 out of 2 of the test mice at dilutions of 10^5 , 10^6 , and 10^7 , none of the animals injected with 10^8 , and one of those injected with 10^9 dilutions died. A third mouse was held at -20°C for 150 days before titration of spleen with the results that 2 out of 2 test animals injected with 10^5 through 10^7 dilutions died and one of each pair injected with 10^8 and 10^9 died of proven plague. One can hardly draw conclusions from an experiment on three mice although the results certainly indicate good, although perhaps somewhat erratic, survival of plague organisms in animals held at low temperatures for periods of a month and a half to five months.

Several secondary observations made during the course of these experiments seem worthy of mention. There seems to be a rather constant number of organisms per milligram of spleen from mice dead from acute plague. When spleens from 32 mice were tested by titration, it was found that 23 killed one or more of the test animals when they were injected subcutaneously with the equivalent of 1 ml of a 10^9 dilution. Eight of the nine spleen emulsions that failed to kill at this dilution did so on the next lowest dilution.

From the several hundred mice that died of known plague and were examined by autopsy a range in weight of the spleen was found from less than 100 mg. to more than 400 mg. The average weight was somewhat more than 200 milligrams. In most instances the classic picture of a greatly enlarged, very dark spleen was observed; but several instances were noted in which animals, dying after the same time interval as those with the typical picture, showed small spleens of normal color. To our great surprise, these few spleens when titrated still killed the test animals in the same range of high dilutions as did the spleens from animals showing the expected gross pathology.

An interesting calculation can be made using the average spleen weight and figures we obtained by titration of spleen emulsions. This would indicate that there are considerably in excess of 20,000,000,000 L.D.₅₀ doses in the spleen of a mouse dying of acute plague after infection with the strain we studied. In view of the very high number of organisms demonstrated in the spleen, the presence of the extreme terminal septicemia is readily understood. Sections of the spleen stained by a modified Wayson's stain show large numbers of plague bacilli particularly in the spaces present because of the extreme edema.

During these studies, which were carried out over a period of a year, there were three occasions on which the titrations from all types of mice (fresh dead, refrigerated, frozen, etc.) showed unexpectedly low end-points. It was noted that these lower end-points had all resulted when the titrations had been carried out during periods of extremely high summer temperatures. These temperatures could not have affected the holding temperature of the mice, and one would hardly expect that the brief period of time they were exposed to a room temperature between 32° and 36°C during grinding, dilution, injection, etc. could have had any effect since peptone was used as the diluent, and the organisms will grow in this material at these temperatures and at 37°C. We postulated that the higher temperature, in some unknown manner, increased the resistance of the host animal to infection. With the advent of cooler weather this hypothesis was tested by running duplicate pairs of mice for each dilution and maintaining one set at room temperature and the other at 34° to 35°C. Unfortunately, however, we have as yet been unable to show any definite effect of temperature on increasing the rate of survival following plague inoculation.

As would be anticipated, the time interval between inoculation and death of the mice varied with the dilution of the inoculum. If the treatment of the original mouse is not considered as a variable, 120 mice per dilution were inoculated with the equivalent of 1 ml for each dilution from 10⁵ through 10⁹. The mice dying from inoculation with each dilution gave average times to death as follows: 10⁵, 2.75 days; 10⁶, 3.3 days; 10⁷, 4.48 days; 10⁸, 4.57 days; and 10⁹, 5.95 days.

Summary and Conclusions

The survival of plague organisms in the spleens of infected laboratory mice was investigated for intervals and conditions that might be adaptable for field studies of sylvatic plague. The study was carried out by comparing the end-points of serial dilutions from weighed spleens of: (1) Mice that just died of plague, (2) mice that had died of plague and been held at 5°C for four

days, (3) mice that had died of plague and been held on dry ice (-78°C) for four days, (4) mice that had died of plague and been held at -20°C for seven days, and (5) mice that had died of plague and been held at -78°C for seven days. The comparison was made by injecting mice with the equivalent of 1 ml of each dilution from 10^5 through 10^9 . There was no significant difference in the end-points of the titrations for fresh dead mice and mice that had been held for four days at either refrigerator or dry-ice temperatures. When the source mice were held for seven days at either -20° or -78°C there was a reduction in end-point of from between one half to one decimal dilution.

In this study, which involved more than 600 mice, an average spleen weight of slightly more than 200 mgms was found for mice dead of acute plague. Titrations showed that the average L.D.₅₀ dose was the equivalent of 1 ml of a 10^9 dilution per mgm of spleen emulsion.

On several occasions spleens were found that were normal in appearance and in weight but nevertheless showed the same high concentration of organisms, when examined by titration, as did the greatly enlarged, dark spleens typical of plague infection in mice.

There was a correlation in time of survival with the dose of organisms injected. Animals dying from the injection of 10^5 dilutions survived, on the average, 2.75 days whereas those mice which died from the injection of 10^9 dilutions survived, on the average, 5.95 days. The mice receiving doses between these two extremes showed times of survival that were also between the above time intervals.

It is concluded that there would be no significant loss of plague organisms from animals collected in the field and held at temperatures of an ordinary refrigerator or on dry ice for periods of four days. If the mice were held at the temperature of a deep-freeze or on dry ice for one week, some slight loss would occur; but this would not be sufficient to preclude the demonstration of plague bacilli if the animals had been infected.

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