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THE SURVIVAL OF *BACTERIUM TULARENSE* IN LICE AND LOUSE FECES[1]

ROGER D. PRICE[2]

This paper presents the results of the first phase of a proposed study of the inter-relationship of the human body louse, *Pediculus humanus humanus* Linnaeus[3], and the tularemia organism, *Bacterium tularense*. The studies dealt with the determination of the longevity of *Bact. tularense* in lice killed immediately after infection, in infected lice allowed to starve to death, and in louse feces, each of the foregoing stored at various temperatures and relative humidities.

Hopla (unpublished, 1950) performed some preliminary experiments on body lice and tularemia, in which he found that infected lice maintained on normal rabbits retained the infection as long as three weeks and that unfed lice were still infected after six days, with a maximum infection of 27,000,000 organisms per louse.

MATERIALS AND METHODS

The body lice used were of the rabbit-adapted strain developed by Culpepper (1948) at the Orlando, Florida, laboratory of the Bureau of Entomology and Plant Quarantine, and were maintained essentially according to the methods recommended by that worker.

The *Bact. tularense* strain used throughout the study was the fully virulent Sm strain having an LD_{50} of $10^{-9.5}$ of a standard suspension for white mice (Downs and Woodward, 1949). A standard suspension of Sm organisms consisted of a 24-hour slant culture suspended in saline and adjusted to a turbidity of 24 per cent light transmission in the Coleman spectrophotometer at wave length of 600.

The agar used for the cultivation of this organism was glucose-cysteine blood agar (GCBA) prepared essentially after the method of Downs *et al.* (1947). This agar was modified slightly to contain a final 1:1,000,000 dilution of gentian violet. By preliminary experimentation it had been determined that this would reduce any possible gram-positive bacterial contamination but would not affect the growth of *Bact. tularense* in any abnormal manner.

The lice and feces were stored at four different temperatures as follows: 37°C., 29°C., 20°C., and 4°C. For each temperature they were stored at three different relative humidities (100, 50, and 0 per cent). The method of controlling

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[3] Hopkins (1952) calls attention to this as the correct name of the louse.

the relative humidities was the sulfuric-acid method as described by Solomon (1951).

A method of louse infection following very closely the chick-skin technique described by Fuller *et al.* (1949) was used. In the preparation of the blood-Sm meal, an attempt was made to establish a meal containing as near 1 billion Sm organisms per ml. as possible. This was done by mixing 6.0 ml. of defibrinated normal rabbit blood with 2.0 ml. of a 10° Sm standard suspension. From the resulting 8.0 ml. of the mixture, 1.0 ml. was pipetted off and used for making serial ten-fold dilutions to 10^{-7}; duplicate plates of 0.1 ml. per plate were made at the 10^{-6} and 10^{-7} levels. This served as a check on the actual number of organisms present in each blood meal.

The removed chick skin was tied over the end of a glass tube, sealed with paraffin, and placed in a beaker containing the blood-Sm mixture and a few glass beads. Lice, consisting mostly of mixed adults starved 8–12 hours, were fed in groups of 75–100 for a period of 20–40 minutes, depending upon how readily they engorged. After feeding, the lice were removed to sterile distilled water, washed briefly for a total of 3–5 minutes in several changes of water, blotted dry on sterile toweling and treated thereafter according to the manner in which they were to be stored.

Small vials were used for all storage. The method of killing the lice consisted of touching each infected louse quickly on the head with a red-hot needle. These killed lice were stored five per vial at the different temperatures and relative humidities.

The method of collecting louse feces was determined by the subsequent conditions of storage. Feces were collected from 10 lice per vial for the 24-hour period immediately following infection. Lice were allowed to defecate for this period under the conditions of temperature and relative humidity in which the feces were to be stored except that, because lice will not defecate at 4°C., all collections for that temperature were made at 20°C. and the corresponding relative humidities. Two methods of fecal collection were employed. A special fecal collector was used to collect feces at 50 and 0 per cent relative humidity. It consisted of a piece of fine-mesh nylon gauze tied over one end of a short piece of glass tubing. This was suspended gauze-end down in a vial and infected lice were placed in the open end. The feces fell through the gauze to the vial floor. For collection of feces at 100 per cent relative humidity, a single-layered disk of bibulous paper was placed in the bottom of the vial and the lice were allowed to defecate directly on this.

The lice from which the feces had been collected were then starved. After the 24-hour defecation period, these lice were removed to new vials and stored 5 per vial under the same conditions at which they had defecated. Only at 4°C. was it necessary to use live lice that had not been allowed to defecate.

The combination of four temperatures and three relative humidities involved twelve different test situations. Under each of these twelve conditions at least 5 vials each of killed lice, starved lice, and louse feces were stored. This experiment, testing all 36 conditions, was gone through once and then completely repeated. The only difference in the two lay in the fact that the first time there were 5 lice stored per vial with feces collected from 10 lice per vial, whereas in the second experiment 10 lice were stored per vial with feces being collected in the same manner as before.

For determination of the presence of *Bact. tularense* in the stored materials, a method of white-mouse inoculation was used. The material of each vial was ground as a unit in 1.5 ml. sterile normal saline with a sterile mortar and pestle. From this, 1.0 ml. was used to inoculate two mice intraperitoneally with 0.5 ml. each. This procedure was altered only on the day on which the lice were infected. Preliminary experiments on louse contamination had showed that only on this day could a reliable quantitative count be made on the Sm organisms in the lice. Thus, the lice on this day were ground in 2.5 ml. sterile saline. This was considered as a 10° suspension and 1.0 ml. of it was withdrawn and used to make serial ten-fold dilutions to 10^{-4}. Duplicate GCBA plates with 0.1 ml. per plate were made for 10°, 10^{-2}, and 10^{-4}. 1.0 ml. of the original suspension was used to make the usual mouse inoculations. Each mouse was observed twice daily and an autopsy made at the time of death if the mouse died within twelve days after the inoculation; mice surviving twelve days were sacrificed then and autopsied. Spleen and heart plate cultures were made for each mouse, and the lymph nodes, spleen, and liver were observed for gross evidence of tularemia. The plate cultures were observed after 24–48 hours for positive growth; if negative, they were allowed to incubate an extra 48 hours. The killed lice, starved lice, and louse feces were tested at varying intervals, depending on the observed trend of the survival of the organisms.

RESULTS

The main experiment involved eight separate preparations of the blood-Sm meal; preliminary experiments necessitated the preparation of four other of these meals; one control experiment required another. Thus, over all, there were thirteen separate chick-skin preparations and twelve infections of lice. In order to determine just how similar these infections were quantitatively, a duplicate plate count was made for the original blood-Sm meal and also for the newly infected lice. Plate counts were made possible for the lice only because the contamination encountered was diluted out at the 10^{-4} dilution, whereas the tularemia organisms were still present. Subsequent plate tests for organisms in stored lice met with consistent failure, due apparently to the increased contamination and decreased number of *Bact. tularense*. This contamination was not even eliminated when lice were treated with a variety of disinfectants before storage.

A comparison of the initial quantitative counts obtained in the thirteen blood-Sm preparations showed a relatively close similarity, the number of organisms ranging from a maximum of 1460×10^6 per ml. to a minimum of 460×10^6 per ml. with an average of 1045×10^6 per ml. Initial louse infection showed a maximum of 2750×10^3 organisms per louse and a minimum of 275×10^3 organisms per louse, the average being 1151×10^3 organisms per louse. Since it

TABLE 1

Longevity of Bacterium tularense *in killed lice, starved lice, louse feces, and blood on bibulous paper (in days)*

SUBSTRATE	RELATIVE HUMIDITY	EXPERIMENT 1				EXPERIMENT 2			
		37°	29°	20°	4°	37°	29°	20°	4°
	%								
Killed lice	100	4.0	4.0	7.5	22.0	3.0	5.5	17.5	25.5
	50	0.5	7.5	17.0	17.0	7.0	11.5	28.0*	29.0*
	0	2.0	14.5	17.0	41.0	4.0	17.0*	25.0*	45.5
Starved lice	100	2.5	6.5	8.0	28.0	3.0	5.0	14.0	22.5
	50	3.0	4.5	17.0	21.5	8.5	3.0	18.0	33.0*
	0	4.0	4.5	32.5	31.5	1.5	11.5	34.5	38.0*
Louse feces	100	1.5	4.0	10.5	20.5	2.0	1.0	3.5	23.0*
	50	9.5	7.0	17.0*	41.0	9.5	14.0*	26.0*	53.0*
	0	13.0*	20.0*	17.0*	50.0*	3.5	22.5	26.0*	53.0*
Blood on bibulous paper	100	No test made with Experiment 1				4.0	10.5	16.0	32.5
	50					0.5	0.5	24.5	49.0*
	0					0.5	0.5	9.0	32.5

* No final negative test.

was the original intent of this experiment to present the lice with a blood-meal containing 1 billion organisms per ml. in the anticipation of getting an average louse infection of 1 million organisms per louse, these quantitative counts showed these conditions were fairly well met.

The results of the main body of the work are given in Table 1. The data shown were arrived at by taking the mid-point between the last positive test and the first negative test. In the cases indicated by asterisks, where unfortunately no negative test was obtained after the last positive, two days were arbitrarily added to the last test. It was found in this respect that infection of lice and feces sometimes showed a very inconsistent tendency as the end-point was approached; occasionally one or two negative tests would be followed by a weakly positive test. Table 2 gives a comparison of the arithmetic and geometric means for the various main effects studied.

As a check to see if the lice were contributing anything to Sm longevity, the survival of *Bact. tularense* in blood alone was tested. The usual blood-Sm mixture was prepared and pieces of bibulous paper 5 by 10 mm. in size were dipped in this mixture and stored singly in vials under the various temperatures and relative humidities. According to Buxton (1947), a female louse starved 6–12 hours will take in approximately 1.0 mg. of blood. Because 10 lice were used per vial in experiment 2, it was assumed they would take in at most approximately 10 mg. of blood. This particular size of bibulous paper was found to absorb approximately 8–10 mg. of blood. The stored pieces of paper were tested at intervals in the same manner as the lice had been, each piece being ground in 1.5 ml. sterile saline and 0.5 ml. of this inoculated into each of two mice. A quantitative

TABLE 2

A comparison of the means of the main effects (in days)

MAIN EFFECT	ARITHMETIC MEAN	GEOMETRIC MEAN
Experiment 1	14.68	9.62
Experiment 2	18.00	11.57
Killed lice	15.52	10.33
Starved lice	14.83	9.75
Louse feces	18.67	11.66
37°C.	4.55	3.45
29°C.	9.08	7.05
20°C.	18.66	16.38
4°C.	33.05	31.06
100% rel. humid.	10.18	6.71
50% rel. humid.	16.79	11.74
0% rel. humid.	22.04	14.93

TABLE 3

Results of statistical analysis

SOURCE OF VARIANCE	d.f.	σ^2
Total	71	0.2104
Experiments (E)	1	0.1168
Humidities (H)	2	0.7613
Substrates (S)	2	0.0376
Temperatures (T)	3	3.1334***
E × H	2	0.1185
E × S	2	0.1266
E × T	3	0.0060
H × S	4	0.1985**
H × T	6	0.0666
S × T	6	0.0654
E × H × S	4	0.0130
E × H × T	6	0.0601
E × S × T	6	0.0206
H × S × T	12	0.0475
E × H × S × T	12	0.0517

Asterisks denote levels of significance:
*** $P < .001$.
** $.01 > P > .001$.

count at the time of absorption showed an initial count of 6 million organisms per piece of paper. The results of this experiment are shown in Table 1.

DISCUSSION

The data as presented in Table 1 were subjected to a four-factor analysis of variance, the factors tested being experiments (E), humidities (H), substrates

(S), and temperatures (T). A log transformation of the data was used after coding by multiplying each value by 10. The results of the analysis are shown in Table 3.

From this analysis it is readily seen that the factor of temperature was highly significant in contributing to the longevity of the organisms in the various substrates; the colder the temperature, the longer the *Bact. tularense* survived.

Humidities did not give a statistically significant difference. They did, however, show a trend in that direction, and, had a sufficient number of replicate tests been made, would presumably have reached the realm of significance. The trend lay in the direction of a longer survival at lower humidity, as evidenced by a mean survival of over twice the time at 0 per cent relative humidity as at 100 per cent humidity.

There was no significant difference shown among the three substrates or between the two experiments. The only other point at which significance appeared was in the humidity-substrate interaction. Undoubtedly the factor contributing most to this was the feces-humidity interaction. Feces, when collected at 100 per cent relative humidity, showed the shortest survival of all groups, whereas feces collected at the two lesser humidities showed the two longest survivals. This may have been due to the fact that feces in the dryer conditions dried very rapidly into small pieces and in this manner mechanically served as a form of protection to the tularemia organisms in the feces. Whatever the cause may be, it is interesting to note that consistently feces at the lower humidities, especially at 0 per cent relative humidity, showed the longest survival of the tularemia organisms. Along this same line, Francis and Lake (1922) found that the feces of tularemia-infected bedbugs, deposited on filter paper and subsequently dried, contained virulent organisms after 20 days, although they would be thought to be much more susceptible to desiccation and destruction in the tiny specks of dried feces than in the body of a killed louse.

When the length of survival of a starved louse is considered, some explanation can be obtained as to why survival of the organisms in them was little different from that in killed lice. According to Buxton (1947), starved lice at 30–35°C. will all die within 3 days and at as low as 10°C. all lice will die within 7 days. Half the lice will be dead usually in about half this time. Thus, essentially the only difference between starved lice and killed lice would be that the starved lice had had opportunity to void themselves of a considerable portion of the material in their digestive tract. This had no apparent effect in qualitative reduction of Sm longevity. Even if *Bact. tularense* were capable of maintaining itself in living lice, as the results of Hopla (1950) seemed to indicate, the starved lice did not survive long enough to make the difference evident. At the 4°C. temperature, starved lice ceased activity and became even more comparable to killed lice.

The saturated humidity created adverse conditions in that it was conducive to mold growth on the lice and feces at all temperatures but the lowest one. Even if the substrate escaped the mold, the high humidity made the substrate soft and spongy. Contrasted to this, survival of *Bact. tularense* in blood on paper was longer at saturated humidity at the two higher temperatures, with a tendency for the humidity effect to be negated at the lower temperatures. Lice and especially louse feces must have guarded the tularemia organisms against at least complete desiccation at these higher temperatures. The greatest point of contrast was found at 29°C. and 0 per cent relative humidity where the organisms on paper survived 0.5 days and in feces 20 and 22.5 days.

A glimpse into the quantitative number of organisms per louse was given by the initial counts per louse, a few successful subsequent counts on stored lice, and the average day of death (A.D.D.) of inoculated mice. These indicated an initial infection approximating 1 million organisms per louse; the rapidity of decrease after that was proportional to the temperature, the higher the temperature, the more rapid the decrease. The mouse A.D.D. often indicated that as the organism neared its end-point, organisms occurred in very small and inconsistent numbers. The whole trend was that of a dying off of *Bact. tularense* with environment determining the rate.

The implications of these experiments are quite clear. Lice under normal circumstances would probably undergo most of their existence within the temperature range of 20–30°C. and at a humidity predominantly lower than 100 per cent. This being true, if lice should have opportunity for a single infective meal from an individual suffering from tularemia, they would potentially be capable of spreading the disease for from 3–4 days up to several weeks even if they received no subsequent nutrition. The bodies of lice killed immediately after feeding or of lice starved to death, along with the feces of the latter, after lying unnoticed for a time, could accidentally come in contact with a suceptible individual and cause the disease. However, body lice in nature are host-specific to man, and until the time when the infectious dose of *Bact. tularense* for man is known and when adequate quantitative data are available for organisms in dead lice and feces, this method of louse infectivity to human beings will have to remain hypothetical.

SUMMARY AND CONCLUSIONS

1. Once a body louse, *P. humanus humanus*, became infected with *Bact. tularense*, both the louse and its feces harbored the tularemia organism for varying periods ranging up to 53 days, depending upon the environmental conditions.
2. Temperature was the greatest single contributing factor to longevity of the organism in the louse or louse feces, with lower temperature causing greater survival.
3. Humidity showed a tendency toward playing a significant part in survival, especially at higher temperatures; the lower the humidity, the longer was the survival.
4. No statistically significant difference was noted concerning longevity of *Bact. tularense* and the louse substrate. Louse feces, however, at low humidity, gave remarkable survival.

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