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CHRONIC SHEDDING TULAREMIA NEPHRITIS IN RODENTS: POSSIBLE RELATION TO OCCURRENCE OF *Francisella tularensis* IN LOTIC WATERS

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Abstract: Contamination of streams by *Francisella tularensis,* a fastidious pathogen, was discovered by Miller in Russia.[14] Subsequently that contamination was found to be the source of extensive human outbreaks, and to occur as well in North America. Circumstantial evidence supports a hypothesis that infected voles are responsible for the contamination, but when freshly isolated *F. tularensis palaearctica* is inoculated parenterally, only acute illness and death result whereas long-term contamination of streams would seem to demand a more chronic process. Laboratory studies have demonstrated that voles have an apparent predilection to tularemic nephritis when partially immunized before parenteral infection, but also when naive voles are infected orally. Associated chronic bacteriuria would seem to fulfill requirements for protracted contamination of watersheds.

INTRODUCTION

Presence of *Francisella tularensis* in streams (lotic waters) in widely separated areas of the Northern Hemisphere has been recognized for many years.[14,18] Water-borne tularemia has occurred in extensive epidemics in Europe[21] and in sporadic infections in North America.[10,15] It has also been a cause of severe epizootics among pen-raised beavers in North America.[3] Certain streams are known to contain the bacteria during various seasons over many years, and this relatively constant contamination has led to conjecture that the bacteria reproduce in aquatic substrates or in poikilothermic aquatic fauna.[18] Other circumstantial evidence links tularemia in rodents to contamination of water,[9] but the direction of transmission of bacteria, whether from water to rodent, the reverse, or both, has not been established.

Detection of bacteriuria in a naturally infected vole (*Microtus pennsylvanicus*) prompted the present study to determine whether chronic shedding infection could be produced in rodents experimentally.

MATERIALS AND METHODS

Animals: Beavers for experimental use were obtained from a local beaver farm.[1] Voles for experiments were taken from a laboratory-reared stock of *M. pennsylvanicus,* an indigenous species in western Montana. The size of the colony does not afford large numbers of animals of uniform age or sex. Mice were from Rocky Mountain Laboratory (RML) stock of Swiss mice known to carry some enzootic viruses and parasites.

F. tularensis vaccine: The "live" vaccine was obtained in July, 1956 from Dr. N. J. Olsuf'ev via Dr. K. F. Meyer, then of the Hooper Foundation, San Francisco. It is prepared from the Gaisky No. 15 culture, used on a large scale for vaccination of man in the U.S.S.R.[23] Presumably, since it is of Old World origin,

[1] Courtesy of Mr. Jerry Milligan, Weaver Beaver, Inc., Hamilton, Montana 59840.

the culture is the type B[11] or *F. tularensis palaearctica* which is of lower native virulence than type A (*F. tularensis tularensis*.[16] Virulence has been further reduced by repeated subculture (attenuated virulence = AV). This organism is lethal to white mice by the subcutaneous (s.c.) route only when inoculated in doses of thousands of viable cells. Mice are much more susceptible when inoculated by the intraperitoneal (i.p.) route, but the organism is not pathogenic for cavies or rabbits by either route. Vaccine was prepared as a suspension, in 0.85% saline, of a 1-day culture on cystine-glucose-rabbit blood agar (CGBA). The suspension was brought to a standard concentration by the method of Francis and Felton.[6] After it had been used as a vaccine, the viable count was determined by plating on CGBA, and mice were inoculated with serial decimal dilutions of the suspension as a control on virulence.

Challenge cultures: The culture used for most experimental exposures was *F. tularensis* (*F.t.*) W-11 isolated at RML from the urine of a naturally infected vole. (Isolation described in "Results".) Virulence of the organism soon after isolation corresponded to that of *F. tularensis palaearctica,* i.e., 1-10 (1 "dex"[2]) organisms inoculated s.c. constituted an LD_{50}[22] for white mice, cavies and voles, and 7 dex viable cells were lethal for domestic rabbits. Average time to death in animals that received minimum lethal doses s.c. was 6 days for voles and mice, 10 days for cavies and 12 days for rabbits. The culture was maintained by freeze-drying. Virulence was tested repeatedly when the culture was reconstituted; no evidence of degradation was found.

Culture *F.t.* 0-362 was used in some tests. It was used as a fresh isolate from a local stream. Virulence tests established that it, too, was typical *F. tularensis palaearctica.*

Creek water was used for preparing dilutions of culture suspension. The water was obtained from a local stream known to have been contaminated with *F. tularensis* on numerous occasions; but when used for the experiment it was free of the bacteria. The water was filtered through a Millipore filter (0.22 μm) before use.

In feeding experiments, water was furnished to the voles *ad libitum* in rubber stoppered bottles with stainless steel tubes. Preliminary tests established that mature voles held in individual glass jars at an ambient temperature of 21 C and about 35% relative humidity consumed an average of 17 ml of water in 24 h. A 20 ml volume of bacterial suspension in water was offered to each vole daily through the period of exposure. Bacterial concentrations are expressed as dex viable bacteria/ml.

Samples of water remaining in the feeding bottles at 24 hours were retrieved and titrated in mice. Ordinarily the titer was diminished by less than 1 dex at 24 h.

RESULTS

Observations of carrier and nonfatal tularemia in rodents

During an investigation of tularemia in farm-raised beavers,[8] serum samples were obtained from many of the animals. One sample from an unvaccinated mature beaver, which had been ill and recovered after antibiotic treatment, had an agglutination titer of 1280. Similar treatment had failed to cure other beavers in the same epizootic.

A second observation was considered highly significant at the time it was made. A large population of voles near Freedom, Wyoming, was reported to be undergoing decimation by disease in 1963. Agents of the Bureau of Sports Fisheries and Wildlife, USDI, collected live voles and shipped them to the RML where they were held in individual jars for observation. Six of seven live animals in the first shipment died of tularemia on the eighth

[2] Dex = order of magnitude[8]

day. Because of the uniform time of death, it was assumed that those voles had become ill by cannibalizing the carcass of an infected pen mate before arrival. Cannibalism is a common method of transmission of tularemia among voles, both in captivity and in the wild.[24]

The surviving vole from the first shipment, and nine that comprised a second shipment, were put in individual metabolism cages periodically, and urine was collected. Urine was inoculated into white mice by the i.p. route and urine from one vole (W-11) caused death of the mice from tularemia. Urine from the same vole 3 days earlier, and 3 days later, did not infect mice. The animal later developed a papilloma of the lower lip and died after 18 months in captivity.

Failure of the vole to succumb to tularemia was considered, at the time, to cast doubt upon the validity of isolation from its urine. Although careful review of circumstances failed to substantiate those doubts, it was reluctantly concluded that undetected contamination from an unknown source must have occurred.

Chronic infection in vaccinated beavers, voles and mice inoculated with virulent F. tularensis

For a test of efficacy of vaccines for prevention of tularemia in ranch-raised beavers, a small group of normal beavers was made available and, to expand the test of vaccine, a group of voles was included. Three concentrations of AV vaccine, differing by a total of 5 dex viable organisms, were inoculated into two beavers and two voles each by the s.c. route. Thirty days later immunity of the animals was challenged by s.c. inoculation of 4 dex viable bacteria of *F. t.* W-11. Beavers were returned to isolation on the donor's ranch 7 days after challenge. Those that survived, and one that died, were not available for necropsy although tissues from the latter were made available for culture.

Voles that survived were killed on day 33 after challenge inoculation. The results are summarized in Table 1.

In this limited trial, distinct vaccine dosage effect was not evident: variations of 5 dex yielded similar results. The best result observed, in both beavers and voles, was survival with no evidence of ill effect. However, some vaccinated animals of both species succumbed to chronic infection. Presence of focal lesions in the kidneys of three voles killed more than a month after challenge was noted at necropsy. None was seen in the chronically infected beavers.

Further comparative tests of beavers and voles could not be done because beavers were not available. In the following experiments, Swiss mice 20-22 days old, and weaned young voles of various ages, were inoculated s.c. with dilutions of suspensions of *F.t.* AV as a preliminary test of susceptibility. The LD_{50} dose in mice was found to be 4 dex viable bacteria, but voles were resistant to >6 dex. Because doses of vaccine containing 2 dex organisms inoculated s.c. were effective in beavers, that dose (approximately 370 viable bacteria) was used. Immunity was challenged 21 days later by s.c. inoculation of 4 dex viable cells of strain W-11.

Urine was collected at various times and tested for presence of the organism (Table 2).

All vaccinated mice and voles survived much longer than the unvaccinated animals but again, as in the previous test, survival was erratic and, seemingly, capricious: 8 of 20 voles and 9 of 20 mice survived to termination of observation while others died as early as the third week. Age and sex were the only known variable among voles; both were uniform in the mice. Only voles developed demonstrable bacteriuria, which occurred unpredictably and inconsistently. Urine of one vole that survived to termination was infectious in two of four tests.

Since voles were found to develop renal lesions or bacteriuria in two experiments, but very irregularly, regular results subject to interpretation were desirable. Therefore, weaned, young laboratory-reared voles were vaccinated with various dilutions of a suspension of

TABLE 1. Results of vaccination (AV) and challenge (W-11) of beavers and voles.

Species Animal #	Vaccine (dex bacterial/ml)	Survived (S) or died (D) - day	Lesions*	*F. tularensis* isolated
Beaver 1	5	S	—	—
Beaver 2	5	D-25	In peritoneum and lung	+
Beaver 3	3	D-18	In spleen, liver, lungs, pleura and pericardium	+
Beaver 4	3	S	—	—
Beaver 5	1	D-8	—	+
Beaver 6	1	D-66	In pericardium, spleen and liver	+
Beaver 7	0	D-7	—	+
Beaver 8	0	D-8	—	+
Beaver 9	0	D-7	—	+
Vole 1	5	S-33	None	+
Vole 2	5	S-33	In spleen and kidney	+
Vole 3	3	S-33	In spleen, scrotum and kidney	+
Vole 4	3	S-33	None	0
Vole 5	1	S-33	In spleen and kidney	+
Vole 6	1	D-5	—	+
Vole 7	0	D-5	—	+
Vole 8	0	D-6	—	+
Vole 9	0	D-6	—	C

*Observed when necropsy feasible. Surviving voles killed at 33 days.
Not done, or not observed, indicated by: —. C = Contaminated culture.
Surviving beaver not killed.

F.t. AV and challenged 11 days later with a uniform dose (4 dex) of W-11 organisms. Urine was tested once for bacteriuria and the results were compared with isolations of the bacteria from tissues (lung, spleen and kidney) of animals that died or were killed. Summarized data are tabulated (Table 3).

This more extensive trial of different doses of vaccine with uniform challenge also failed to demonstrate a consistent pattern of survival or bacteriuria. However, it was evident that even 1 dex bacteria were sufficient to immunize against challenge inasmuch as none of the three voles thus vaccinated died. In one test, bacteriuria occurred but *F. tularensis* was not isolated from tissue. Probably infection was lost in the period between urine test and necropsy.

In another attempt to obtain more consistent results, voles were vaccinated with a uniform dose of 5 dex *F.t.* AV, and 10 days later they were inoculated s.c. with dilutions of a suspension of W-11. All nonvaccinated voles that were inoculated with 1 dex organisms died. Urine was collected weekly from some of the voles; others were not tested. Surviving voles were killed and necropsied 43

TABLE 2. Vaccination (AV) and challenge (W-11) of voles and mice: Survival* and bacteriuria†

Animal #	Voles		Mice	
	Survival days	Bacteriuria	Survival days	Bacteriuria
1	72	2/6	110	0/2
2	110	0/6	27	0/2
3	110	0/6	110	0/3
4	15	0/2	26	0/1
5	37	0/1	26	0/1
6	26	0/5	28	0/2
7	110	0/6	110	0/5
8	15	0/2	22	0/2
9	110	0/6	110	0/1
10	19	0/2	110	0/0
11	110	0/6	110	0/3
12	43	2/4	28	0/1
13	39	1/3	110	0/2
14	110	2/4	28	0/0
15	110	0/6	36	0/2
16	45	1/4	110	0/1
17	74	2/6	110	0/4
18	110	0/6	42	0/1
19	15	0/2	110	0/3
20	43	2/4	28	0/2

* Maximum survival (period of observation): 110 days.
† Test positive/number of trials.

TABLE 3. Voles vaccinated with various doses of AV and challenged with a uniform dose of W-11: Survival and bacteriuria.

Voles* nos.	Vaccine no. bact. dose	Isolation of *F. tularensis from*						No. died	Gross lesions on kidney
		Urine	Tissue	Urine & Tissue	Urine only	Tissue only	Total (all)		
1-7	7	3/7	5/7	3/7	0/7	2/7	5/7	3/7	2/6
8-13	6	1/6	2/6	0/6	1/6	2/6	3/6	0/6	1/6
14-20	5	3/7	4/7	3/7	0/7	1/7	4/7	1/7	2/7
21-25	4	1/6	1/6	1/6	0/6	0/6	1/6	0/6	1/6
26-31	3	2/6	1/6	1/6	1/6	0/6	2/6	2/6	1/4
32-39	2	0/8	4/8	0/8	0/8	4/8	4/8	1/8	0/8
40	1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1
41-43	<1	2/3	2/3	2/3	0/3	0/3	2/3	0/3	2/3

* Several voles were lost in some groups as a result of fighting.
† Number positive/number tested.

days after challenge inoculation. Occurrence of bacteriuria, mortality and kidney lesions are summarized in Table 4.

In this experiment, as in the previous one, reaction to infection varied in an irregular way, i.e., no pattern of dose response was discernible in mortality of bacteriuria. There was marked variation in duration of bacteriuria: some samples were infectious only the first week after challenge; others for the four weeks of test. Neither the incidence nor duration could be correlated with challenge dose.

Results of vaccination and challenge of voles yielded unpredictable results in regard to any individual animal; nevertheless, chronic disease, often with recovery and often with bacteriuria, was produced in the groups of voles with various doses of vaccine and challenge organisms.

TABLE 4. Voles vaccinated with a uniform dose of AV and challenged with various doses of W-11: Survival and bacteriuria.

Voles nos.	Challenge dose—No. bact. dex	Isolation of *F. tularensis* from:		No. died	Gross lesions on kidney
		Urine*	Tissue		
1-10	9	4/4	8	6	2
11-20	7	1/2	10	9	0
21-30	5	2/5	7	5	1
31-40	3	2/3	9	9	0
41-50	1	3/6	8	6	3

* No. voles with positive urine at least once/No. of voles tested.

Production of bacteriuria tularemia in voles by ingestion

In various experiments we had found that large numbers of virulent *F. tularensis* cells are needed to infect mice and voles by gavage. The fact that smaller numbers failed to cause detectable infection suggested the possibility that they might partially immunize and that a subsequent larger dose might then cause nonfatal or chronic infection with bacteriuria. To test that possibility, normal weaned voles of various ages were given, for drinking, creek water to which were added known numbers of viable W-11 on 4 successive days per week for 5 weeks. Concentrations of bacteria offered to 4 groups (of 10 voles, each) were 2, 3, 5 and 7 dex/ml initially. Voles that survived exposure in each group were given concentrations 1 dex greater each successive week.

Mortality occurred rapidly in voles given concentrations of bacteria 5 dex or greater and there was no evidence that prior exposure to nonlethal concentrations exerted an ameliorating effect. However, seven voles developed bacteriuria; two of them had infectious urine on two occasions a week apart when exposed to bacterial concentrations of 3 and 4 dex. All of them died after exposure to the 5 dex concentration. Successive decimal increments of bacterial concentrations in drinking water were separated by periods of only 3 days in this test and, therefore, a vole with bacteriuria would not be detected until after it had been exposed to the next higher concentration.

The next experiment was similar except that a test observation period of 8 days intervened between successive incremental exposures. Thirty-six voles were exposed; six of them developed bacteriuria and they died on 1, 1, 3, 3, 4 and 5 days, respectively, after passing known infected urine. Renal lesions were not seen in any of the 36 animals at

necropsy. *F. tularensis* was isolated from spleen, kidney, or both of all, except one which was autolysed and yielded a heavily contaminated culture.

Six of 27 voles exposed to water contaminated with a freshly isolated culture, *F.t.* 0-362, on alternate weeks also developed bacteriuria. One of them survived 8 days, and another was killed in apparently normal condition 15 days after initial detection of *F. tularensis* in the urine.

Freshly isolated cultures of type B, the type found in water, as well as those of type A, are lethal to mice and cavies in doses of 1-10 viable organisms inoculated parenterally. Voles, tested with cultures of degraded virulence, are slightly more resistant than mice. Whether voles ever survive inoculation of minimal numbers of virulent bacteria is uncertain, but in a test of that question, we inoculated several decimal dilutions of a suspension of *F.t.* 0-362 into mice and voles by the s.c. route. Voles and mice that survived inoculation with terminal dilutions of suspensions were challenged, along with normal controls, by s.c. inoculation of 4 dex of the same culture 2 weeks later. None survived. A similar test of culture W-11 yielded similar results. We infer that parenteral introduction of even one infectious virulent organism results in progressive fatal infection rather than abortive immunizing infection.

DISCUSSION

Water contaminated with *F. tularensis* is a common cause of human infection in some parts of the Northern Hemisphere[21] and may be more common than is generally recognized in other large areas.[10,20]

In a study of *F. tularensis*-contaminated waters in Montana and Idaho, Parker *et al*[18] investigated the possibilities of propagation of the organism in poikilothermic aquatic vertebrates and in mud. They found that tissues of the vertebrates did not become infected in inoculated water although intestinal contents of one trout were infected, probably because the organism was "present in ingested water." The bacteria were demonstrated in water and mud samples stored at 7 C for as long as 14 weeks, and tests of mud extracts demonstrated capability of the medium to support growth of the organism. They concluded, tentatively, that the organism multiplies in the water-mud medium.

After reviewing the evidence presented by Parker *et al.* we believe that presence of the bacteria in their media for long periods could be explained by survival rather than propagation. Infectiousness of the menstruum seemed to be favored by low temperatures (see also Kartman *et al.*[12]) yet we have been unable to cultivate *F. tularensis* on rich media at the low temperatures to which the water was subjected (J. F. Bell, unpublished). In the absence of any known temperature-compensating mechanism, a hypothesis of propagation of the organism in mud would seem to be untenable.

Circumstantial evidence of the source of contamination at times rather strongly implicates rodents, because surface waters are heavily contaminated in areas where epizootics of tularemia occur.[9] Nevertheless, direct evidence that rodents are responsible for the contamination is lacking, and the source of contamination of water during interepizootic periods is especially puzzling.

Occurrence of infected carcasses in or adjacent to contaminated streams does not establish direction of transmission of the organism. On three occasions we have found tularemia-infected carcasses in a small brook (cross section about 1 square meter) and have been unable to isolate the organism downstream (J. F. Bell and G. J. Moore, unpublished), whereas the same stream has been found contaminated with *F. tularensis* annually (and for long periods) for at least 13 years and no carcasses were detected on those occasions.

Constant or frequently repeated contamination of a stream could be explained if a homiothermic hygrocolic (inhabitant of moist places) vertebrate were chronically infected, and shed the

organism in its urine onto a watershed. There are several candidate species for the role, e.g. beavers, muskrats, and voles of various species. Of these, only the voles are found in habitats adjacent to both large and very small contaminated streams. They, as well as muskrats and beavers, are frequently infected.[9]

In our limited inquiry into presence of *F. tularensis* in urine of naturally infected *Microtus,* one animal was found to shed the organism in its urine. Survival of voles after parenteral inoculation of *F. tularensis* was also demonstrated but only with organisms of diminished virulence or, with virulent organisms, after vaccination. Occurrence of chronic infectious nephritis in voles suggested that the etiologic agent would be found in the urine. In a group of 20 voles vaccinated with AV and challenged 3 weeks later, infectious urine was demonstrated 1 or more times in 7. Six of them died 39 to 74 days after challenge; one survived until killed at 110 days. It is clear that voles exhibit predilection to chronic shedding nephritis, but the experimental infection after vaccination has no known counterpart in nature.

Theoretically, nonfatal chronic infection could be caused by organisms of low virulence as well as by virulent organisms in partially immune animals and when the AV strain was inoculated into mice, one mouse retained the infection for 57 days. There was no enhancement of virulence during residence in the animal, a finding consistent with the observations of Owen *et al.*[17] However, in a large series of tests of freshly isolated *F. tularensis* from a wide variety of sources (C. R. Owen and E. Buker, unpublished) two distinct types of organisms[16] were identified repeatedly, but in each instance only fully virulent types were found; there were no fresh isolates of intermediate virulence, or of virulence lower than that of *F. tularensis palaearctica.*

Another explanation of nonfatal infection was based on a hypothesis that very small doses of fully virulent bacteria might initiate infection but that resistance would develop before serious infection could ensue. However, when freshly isolated cultures were titrated by the s.c. route in mice and voles, survivors at the terminal dilutions were not subsequently immune to challenge by parenteral inoculation of larger doses of the same organism.

Nevertheless, chronic infection has been noted in very susceptible white mice (*Mus*) as well as in the relatively resistant *Rattus norvegicus,*[7] in snowshoe hares (*Lepus americanus*)[2] and in man.[18] Burroughs *et al.*[4] state that tularemia may occur in "latent" form in voles and other rodents, but documentation is not presented.

In view of the fact that cannibalism is common in rodents and is a common cause of spread of tularemia among them,[9,12,24] ingestion of virulent organisms was investigated as a possible mechanism of chronic infection. Results were extremely irregular: most of the voles succumbed to acute infection but some developed nephritis with bacteriuria that persisted for at least 15 days. Dunaeva and Olsuf'ev[5] had discussed the possibility of a latent or chronic course of infection in voles and other species highly susceptible to tularemia. We can offer no explanation for the occasional chronicity of infection when acquired by ingestion, for its erratic occurrence or for apparent limitation of nephritis and bacteriuria to voles when voles and mice were tested under identical conditions. However, in spite of erratic occurrence, a like proportion of bacteriuria in wild voles would make a significant contribution to contamination of the watershed of a brook. In other studies (J. F. Bell and S. Stewart, unpublished) we had isolated *F. tularensis* from 2 of 49 soil samples in the immediate watershed of a brook that is frequently contaminated in excellent and occupied vole habitat.

The observations reported here lend support to a hypothesis that rodents of the subfamily Microtinae are responsible for contamination of streams by *F. tularensis.* Known distribution of *F. tularensis palaearctica* appears to be limited by the distribution of voles. However, voles occur in some places where tularemia

has not yet been found (e.g., Great Britain; North Africa). Hygrocolic species of the genera *Arvicola* and *Microtus* are common, and are very commonly infected where waters are contaminated by *F. tularensis*. During epizootics of tularemia in those species, both lentic and lotic waters are heavily contaminated. However, explanation of persistent contamination of certain streams over many years (to be published) would seem to require continuous or frequent, rather than episodic, renewal of the source. Fatal infections would have to be frequent if carcasses were to serve as a source. Three tests of infectivity of water, downstream from infected carcasses, failed to yield *F. tularensis*. Demonstration of *F. tularensis* in soil, long survival of the organism in mud,[18] and bacteriuria for at least 15 days in voles infected by ingestion, a common method in nature, would seem to satisfy the requirements for persistent contamination of water, although other sources are not necessarily excluded.

Whether tularemia in voles is contracted from contaminated water is uncertain. Although the potential has been demonstrated in the laboratory, and although beavers and humans have contracted the disease frequently from that source, concentrations high enough to infect voles have not been detected in our limited quantitative tests of streams in interepizootic periods.

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