ANNUAL REVIEWS

Annual Review of Entomology

Ecology of *Francisella tularensis*

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Annu. Rev. Entomol. 2020. 65:351–72

First published as a Review in Advance on October 10, 2019

The *Annual Review of Entomology* is online at ento.annualreviews.org

https://doi.org/10.1146/annurev-ento-011019-025134

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Keywords

Francisella tularensis, tularemia, ecology, arthropods, natural foci, maintenance

Abstract

Tularemia is a Holarctic zoonosis caused by the gamma proteobacterium *Francisella tularensis* and is considered to be a vector-borne disease. In many regions, human risk is associated with the bites of flies, mosquitoes, or ticks. But the biology of the agent is such that risk may be fomite related, and large outbreaks can occur due to inhalation or ingestion of contaminated materials. Such well-documented human risk factors suggest a role for these risk factors in the enzootic cycle as well. Many arthropods support the growth or survival of the agent, but whether arthropods (ticks in particular) are obligately required for the perpetuation of *F. tularensis* remains to be demonstrated. As with most zoonoses, our knowledge of the ecology of *F. tularensis* has been driven with the objective of understanding human risk. In this review, we focus on the role of the arthropod in maintaining *F. tularensis*, particularly with respect to long-term enzootic persistence.

> Tularemia is a specific infectious disease due to *Bacterium tularense* and is transmitted from rodents to man by the bite of an infected bloodsucking insect or by the handling and dissecting of infected rodents by market men or laboratory workers. (42, p. 1731)

1. INTRODUCTION

During plague surveillance in 1909, an epizootic in ground squirrels from Tulare County, California, yielded bacteria that did not have the characteristic safety pin morphology of plague bacilli (96). The bacterium was quickly cultivated and named *Bacterium tularense* (97). Edward Francis of the US Public Health Service (USPHS), while investigating an outbreak with human cases presenting with ulceration, lymphadenopathy, and fever in Utah residents bitten by deer flies, incriminated *B. tularense* as the etiologic agent and proposed the name tularemia for the disease (42). His comprehensive investigations included isolating the agent from fly-bitten humans, from jackrabbits, and from ground squirrels. Francis also provided experimental evidence for transmission of tularemia by the bites of deer flies, mouse lice, and bed bugs (42). Parker and colleagues isolated *B. tularense* from the Rocky Mountain spotted fever vector, *Dermacentor andersoni* (114), and the related American dog tick, *Dermacentor variabilis*, was incriminated as the source of a human infection in Minnesota (57), thereby formally incriminating ticks, in addition to deer flies, as a risk to humans. Francis requested that each state's department of health report any case of tularemia and determined that >90% of >6,000 tularemia case reports from 1924 to 1935 were associated with exposure to cottontail rabbits or hares (43), mainly as a result of market production of rabbit meat but also due to rabbit hunting. His specific mention of laboratory workers in his pithy summary of tularemia was included because all six of the USPHS staff working on the newly recognized infection became infected (83), including Francis himself. The ease with which laboratory workers became infected by manipulating cultured bacteria or infected animals (the agent was easily propagated by serially transferring infected tissue homogenates to uninfected rodents) became notorious. UK bacteriologists, after receiving reference cultures from Francis, became infected and decided to cease work with *B. tularense* because of its great hazard (138).

Very soon after Francis' seminal work, Hachiro Ohara in Japan described 10 cases of an acute febrile disease that had been acquired during the skinning of wild rabbits (48). The disease had been noted in the Fukushima area for 20 years but had not drawn attention from clinicians. Rabbit die-offs had been frequently noted by villagers. Definitive proof that rabbits were a source of infection was provided when Ohara's wife volunteered to be exposed: She acquired tularemia after tissues from a dead rabbit were rubbed on the back of her hand. The clinical details provided by Ohara agreed "even in minor details with the details of tularemia" (48, p. 1332), sera sent by Ohara to Francis in the United States agglutinated US *B. tularense* strains, and fresh tissues from a Japanese case sent to Francis produced typical tularemic lesions in laboratory rodents.

Tularemia has had a long history in the former Soviet Union, and much knowledge is contained in their literature, which is in Russian and thus sadly less accessible to most Western workers. Pollitzer (125) provides a comprehensive review of the historical Soviet epidemiological literature, and the reader is directed to that extraordinary book. To summarize from that authority, tularemia was first definitively identified in the Soviet Union when, in 1929, Zarkhi, a clinical researcher in Sverdlosk, sent his own serum to McCoy at the USPHS. Zarkhi had suffered what he suspected was tularemia after necropsying guinea pigs inoculated with bubo contents from patients of a water rat–associated outbreak of a mystery disease. Pollitzer pointed out that the Soviet scientists retrospectively made the connection between outbreaks of so-called Siberian ulcer in the eighteenth and nineteenth centuries (which was originally considered to represent cutaneous anthrax) and tularemia given the low (2%) mortality associated with some outbreaks of Siberian

ulcer; the case fatality rate for anthrax would be much greater. Interpretation of historical outbreaks suggested that tularemia had long afflicted people throughout the vast Soviet Union from west to east and north to south, and thus the infection was very old and not recently introduced, for example, with the liberation by the Soviet fur industry of up to 80,000 muskrats from 1928 to 1945 (28). Muskrats have been associated with tularemia outbreaks across the Holarctic.

2. CHARACTERISTICS OF THE CAUSATIVE AGENT

The genus *Francisella* comprises gram-negative coccobacillary, non-spore-forming, aerobic or microaerophilic bacteria in the order Thiotrichales. Isolates with growth or biochemical characteristics similar to those from humans suffering from tularemia have been made from marine and freshwater fish, brackish water and other environmental sources, and ticks. Non-tularemia patients, often immunocompromised, have also yielded novel isolates that classically group with *Francisella* spp. Whole-genome analyses demonstrate that there are at least a dozen distinct species (24). Three subspecies of *Francisella tularensis* are currently recognized: *tularensis*, *holarctica*, and *mediasiatica*. Tularemia was classified into two types according to distributional, ecological, biochemical, and pathological characteristics (107). Those causing severe disease, often tick transmitted and restricted to North America, were Type A (now known as *F. tularensis tularensis*). A Holarctic form, Type B (*F. tularensis holarctica*; *F. tularensis palaearctica* in some older literature) caused episodic epizootics in beavers, muskrats, and arvicoline rodents and milder disease (74, 115). *F. tularensis mediasiatica* was isolated from rodents in Central Asia, but there are no reports of human cases. *F. tularensis mediasiatica* has apparently been isolated from diverse ticks from most metastriate genera as well as *Ixodes persulcatus* (149). *Francisella novicida* has been considered a subspecies of *F. tularensis*, but this bacterium is actually a distinct species (80). *F. novicida* is an opportunistic pathogen, mainly of immunocompromised or elderly individuals exposed to environmental sources; isolates have only been made from salt or brackish water, never from animals or arthropods. However, it is highly virulent for experimental rodents, causing typical tularemia, and can be acquired by feeding, survive transstadially, and replicate within and be transmitted by the bites of *D. andersoni* (132).

Genetic typing methods confirmed the phenotypic distinctions and identified distinct lineages within Type A and Type B. Multilocus variable number tandem repeat analysis (MLVA) split the former into A.I. and A.II. Virtually all isolates from the eastern and central United States were A.I., and those from west of the 100th meridian were A.II. Whole-genome sequencing demonstrates that there are three lineages of A.I. (17), with A.I.12 being the most widely distributed, suggesting that A.I.12 has an adaptive advantage over the others. Four major lineages are apparent across the Holarctic Type B isolates (144), with considerable diversity of subpopulations and genotypes even within endemic counties in Sweden (76). Newer typing methods using single-nucleotide polymorphisms produce results consistent with MLVA but designate lineages slightly differently (e.g., A.I. is A1).

3. EVOLUTION OF *FRANCISELLA*

The genus *Wolbachia* was erected by Marshall Hertig to describe bacteria that he and Burt Wolbach (who described the rickettsial etiology and pathology of Rocky Mountain spotted fever) found in the reproductive tract of *Culex pipiens* in 1924 (60). Subsequently, a variety of endosymbiotic intracellular bacteria, most identified only by microscopy of stained smears of arthropod tissues, were considered to be *Wolbachia*. *Wolbachia persica* was isolated from *Argas arboreus* ticks collected from an Egyptian heron rookery (140). *W. persica* was pathogenic for guinea pigs, mice, and chicks

but not rats or rabbits. Molecular phylogenetic analyses clearly demonstrated that *W. persica* was not a rickettsial agent but was closely related to *F. tularensis* (102, 103) and not to *Wolbachia pipientis*, the bacterium first identified by Hertig and Wolbach. *W. persica* has been formally reclassified in the genus *Francisella* (85). *Francisella*-like endosymbionts (FLEs) have been identified from most tick genera (32, 54, 71, 103, 133, 137, 141, 142). Early analyses suggested that tick–FLE associations were ancient (103) and thus that *F. tularensis* may have evolved from FLEs. However, cospeciation between *Dermacentor* spp. and their FLEs was not detected (137), and critical virulence genes for *F. tularensis*, such as type IV pili, lipopolysaccharide (LPS), and the type VI secretion system, are present but have become pseudogenes in the FLEs (51). Accordingly, FLEs were acquired by ticks from a virulent *F. tularensis*–like ancestor. If *F. tularensis* did not originate with ticks, then the presumed central role of ticks for maintaining this infection requires critical analysis.

4. EPIDEMIOLOGY OF TULAREMIA

Tularemia is endemic throughout most of Europe, northern and central Asia, and North America. Type B is found in most of Europe, Asia, and North America. Type A is found exclusively in North America; there is one report of its identification in Europe, but this finding remains enigmatic. *F. tularensis mediasiatica* has been identified in limited portions of Central Asia. Although tularemia was not previously thought to be present in the southern hemisphere, in 2011, three human cases were identified in Tasmania, and isolates made from ringtail possums (*Pseudocheirus peregrinus*) were determined by sequencing to group with Type B strains from Japan (sometimes referred to as biovar japonica) (33).

The clinical presentation of tularemia is varied, with six classical forms having been described: ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal, and pneumonic. Such presentations generally relate to mode of acquisition; oculoglandular and oropharyngeal presentations, for example, are the result of conjunctival contamination and ingestion, respectively. Ulceroglandular presentation, with ulceration at the site of an arthropod bite or other portal of infectious entry as well as proximal lymphadenopathy, and glandular presentation, with regional lymphadenopathy, are the most common, comprising 50–65% of all North American cases (40, 154). The other common form, typhoidal, has an acute onset with sore throat, high fevers, chills, and enteric symptoms. No ulcer or portal of entry is evident, nor is there lymphadenopathy. Pneumonic tularemia may comprise primary inhalational tularemia with a respiratory portal of entry (129), exemplified by laboratory accidents (109) in the years before universal laboratory adoption of biosafety cabinets and by large agriculturally related outbreaks (27). However, pleuropneumonia is a common finding in advanced typhoidal tularemia (8, 21, 41) and in 10–20% of ulceroglandular cases. Pneumonic tularemia, presumably primary inhalational, is increasingly being reported in the United States and, in some sites, may be more common than ulceroglandular tularemia (117). In Europe, ulceroglandular and glandular presentations comprise 50–70% of reported cases (e.g., 37, 92).

In Europe and across Russia and the Federation of Independent States (due to the seminal contributions of Soviet scientists, we use the term Soviet Union), the epidemiology of tularemia is diverse, reflecting the diversity of the land, fauna, and people (61). Mosquitoes are strongly associated with tularemia in Sweden (136). Oropharyngeal tularemia is the most commonly reported form in some countries (Bulgaria, Kosovo, Norway, Serbia, and Turkey), thought to be due to contamination of water or food by rodents. Hunting, mainly of hares, is the source of ulceroglandular or glandular infections in the Czech Republic, France, Germany, Slovakia, and Spain. Ticks are not considered to be a common source of infection in Europe (61).

In the Soviet Union, six epidemiological scenarios were recognized (93). (*a*) The first was the meadow–field type, with the vole *Microtus arvalis* as amplifying host and *Dermacentor reticulatus* as the vector and interepizootic reservoir. Risk to people was associated with agriculture and contamination of drinking water. (*b*) The second was the steppe type, with diverse rodents and hares as the amplifying hosts and the vector *Dermacentor marginatus*; agricultural activity and contaminated water created the risk to people. (*c*) The third was the forest type, with the vector *Ixodes ricinus* and its main subadult hosts (*Myodes* spp., *Apodemus* spp.); people were infected during hare hunting, as well as by tick bites. (*d*) The fourth was the floodplain–swamp type. *Arvicola terrestris*, the water rat, was the critical amplifying host, with *Dermacentor*, *Rhipicephalus*, and *Ixodes* ticks as vectors. Mosquitoes could also infect water rats. Humans became infected by hunting water rats. (*e*) The fifth was the foothill–brook type. Water rats were also the main host, with *Ixodes apronophorus* as the vector. [*I. apronophoros* also plays an important role in maintaining Omsk hemorrhagic fever virus in the same sites (79)]. People became infected by the contamination of running water during the summer by water rats. (*f*) The sixth was the desert river type. Hares, gerbils, and *Rhabdomys pumilio* are thought to maintain infection, with risk exclusively to hunters. These Eurasian epidemiologically based scenarios demonstrate the possible great diversity of enzootic cycles that may characterize *F. tularensis* elsewhere.

5. RABBIT FEVER

The perception that Type A tularemia was due to lagomorphs was largely the influence of Francis himself and also of William Jellison of the Rocky Mountain Laboratories, who compiled and interpreted the existing literature on tularemia biology in a seminal monograph (73). Jellison argued that human risk and geographic distribution of North American tularemia was strongly associated with cottontail rabbits (75, 112). Cottontail rabbits have several characteristics that may bias analyses of their role in tularemia epidemiology. They were once a domestic food source, with a value of as much as $5,000,000 in the 1920s (59), and intensively hunted. Rabbits almost always die of Type A tularemia, and their carcasses are easily noted during die-offs. Then, as now, tularemia cases were common in the southcentral states, not coincidentally where people still hunt rabbits (155). Generally, across the United States, hunting has diminished as a sport, and rabbits are no longer popular as food, thus contributing to a reduction in the incidence and prevalence of tularemia (18).

In Japan, tularemia risk is mainly associated with rabbits (104). 93% of 1,358 cases analyzed from 1924 to 1994 were considered to have been due to rabbit exposure. In addition, a large spike in the incidence of tularemia from 1945 to 1955 was attributed to soldiers resettling in their homes eating rabbits as a source of protein in economically challenging postwar times; the incidence started falling dramatically with robust growth in the Japanese economy.

Rabbits and hares clearly have an important role in the epidemiology of tularemia, either as the direct (meat or hunting) or indirect (contamination of the environment by carcasses) source of human infection; they also draw attention to epizootics because their die-offs are easily noted.

6. ECOLOGY OF TULAREMIA: GENERAL COMMENTS

Tularemia usually comes to our attention as a result of epizootics (spillover from local amplification with clusters of human cases), but endemic tularemia is underappreciated. The Midwestern United States reports a fairly constant (limited interannual variation, no more than twofold) number of cases each year (largely tick transmitted), as does the island of Martha's Vineyard, Massachusetts (approximately equal numbers of cases from tick transmission and from inhalation) (94). Sweden

also has constant annual numbers of mosquito-transmitted infection (36), and that country and Finland report more tularemia cases than any other (61). In other countries, clinical reporting might be less of a priority, and thus it is possible that countries other than Sweden and Finland sustain a similar degree of tularemia endemism. Reported cases, however, reflect only an effective epidemiological bridge from an enzootic cycle (endemic transmission) or epizootic (spillover from massive time-limited amplification of the enzootic cycle).

Most zoonotic agents remain in longstanding natural foci with physical and biological characteristics to which they are adapted (116). Human risk is associated with disturbing such natural foci. A scenario for *F. tularensis* perpetuation may comprise a system (metapopulation) of small natural foci, each with a prevalent variant and mode of transmission. This fundamental tenet of the ecology of vector-borne agents asserts that such pathogens are perpetuated within specific sites, often on the order of tens of square meters in area, and that transmission may be continuously detected there for decades or longer. Humans become exposed by stumbling into such a natural focus or when transmission risk becomes more homogeneously distributed over a wide area due to many such foci coalescing as a result of local amplification and spillover. Goethert & Telford (55) identified one such natural focus on Martha's Vineyard and demonstrated that infected American dog ticks were found mainly in a 260-m-diameter site along a long-term transect and that the most genetic variability of *F. tularensis* along the transect was to be found in ticks in that microfocus. Great genetic diversity was apparent on Martha's Vineyard as a whole, with indices of heterogeneity as great as that measured in analyses of *F. tularensis* isolates across the world (53); *F. tularensis* exists there as a metapopulation of isolated microfoci (52). How such microfoci remain genetically discrete on an island of approximately 1,000 km^2 is unclear.

Mathematical models suggest that "The maintenance of indirectly transmitted infections do not require the very large host populations that are needed for directly transmitted microparasite infections" (95, p. 155). Given the virulence of *F. tularensis* for rodents or lagomorphs, with the majority of infected animals dying rapidly, some indirect factor (vector, fomite) is required for the bacterium to persist over ecological time. It seems unlikely that long-term persistence is due to direct contact between infected and uninfected vertebrate hosts, although this mechanism certainly has the capacity to dramatically amplify transmission during an epizootic given that excreta can be infectious and that cannibalism of animals dying from tularemia may result in new infections. Without a persistent reservoir in a vector or fomite, or chronic infection of a longer-lived vertebrate, generation of susceptible hosts by immigration or recruitment would need to at least equal the removal of infected hosts through death.

Is there a main theme for perpetuation (ticks and rabbits, for example), with regional ecological differences being variations on that theme? Even with the limited literature, it is clear that it is not a good assumption that the ecology of Type A is similar to that of Type B. Soviet scientists described six different kinds of natural foci: meadow–field, steppe, forest, floodplain–swamp, foothill–brook, and desert river (106). *F. tularensis* Clades A.I. and A.II. are genetically and generally geographically distinct, suggesting that they occupy different ecological niches or were isolated during glaciation (78). It may be that the diverse sublineages of Type A and Type B (81, 151) might each have differing requirements for maintenance. However, both A.I. and A.II. were isolated from lagomorphs in one discrete site in a Utah fly-transmitted outbreak, demonstrating co-circulation (118). This finding does not preclude more than one kind of natural focus within a geographically discrete area.

6.1. Associations with Arthropods

Although tularemia is considered a vector-borne disease, an obligate role for arthropods, and hematophagous species in particular, in maintaining *F. tularensis* in nature is not axiomatic. Ticks

are considered to be main vectors and likely reservoirs for *F. tularensis* in North America and much of Eurasia (73, 116), but ticks have not been incriminated as being relevant to the enzootic cycle in Scandinavia, which reports more tularemia cases than any other region in Europe (although this is due in part to the likely high proportion of cases that are reported). It is possible that many kinds of arthropods, perhaps even nonhematophagous species, may contribute the perpetuation of *F. tularensis* over the long term in natural foci.

6.2. Associations with Vertebrates

F. tularensis has been isolated or detected from a very large number of animals, including amphibians, birds, rodents, lagomorphs, carnivores, and ruminants (22, 67). Virtually all of these are considered incidental hosts, although if they die of sepsis due to *F. tularensis*, and if there is an appreciable bacterial burden in their tissues, then they may contribute to short-term and perhaps long-term maintenance as long as decomposing tissues can remain infectious under certain conditions. The carcass of a mouse dying of most of the main tularemia lineages may have 9–11 log colony-forming units (cfu) within the spleen or liver (100). Interestingly, rats (*Rattus norvegicus*) can recover from infection, and viable bacteria may be recovered from their tissues for at least two weeks after recovery (30, 31); their role as possible enzootic reservoirs should be critically examined given their global distribution and abundance. Other animals, such as carnivorous mammals or raptors, are typically poorly susceptible to disease (seroconverting with infection) and are excellent sentinels given their scavenging and predator roles (15). An exception is the domestic cat, which is commonly infected [as many as 12% are seropositive in some sites (90)], suffers disease that is not infrequently fatal, and in fact serves as a risk to their owners and to veterinarians (84). Given the abundance of feral cats (87), at least in the United States, their potential role in maintaining natural foci should be analyzed further.

6.2.1. Adaptation to specific animals. Some evidence suggests the possibility that some strains (lineages) of *F. tularensis* are specifically associated with certain mammals. The prevalence of human cases in the United States overlaps with the distribution of cottontail rabbits (112), and Type A strains were most likely to have been isolated from these hosts (81); A.I. strains came from eastern cottontails (*Sylvilagus floridanus*), and A.II. came from desert cottontails (*Sylvilagus audubonii*). However, there does not seem to be any specific relationship between a vertebrate species and lineages of Type B (124). Of course, existing *F. tularensis* strains do not represent a random sampling and reflect convenience samples from clinical cases or from outbreak investigations. Efforts should be made to isolate additional strains from a greater selection of vertebrate species before any conclusions can be made.

6.2.2. Is Type B ecology dependent on rodents? Tularemia risk in both Eurasia and North America has a strong environmental basis. Agricultural activities (hay threshing; water contaminated by rodent excreta or carcasses; trapping or hunting) (3, 61, 116, 134, 145) have been associated with large outbreaks. A large typhoidal (pneumonic) tularemia outbreak in Castilla y Leon, Spain, was associated with farm and harvest activities, as well as contact with voles (3). A prior large outbreak in the same region had been associated with hunting hares, and subsequent genetic analyses of strains isolated from both outbreaks demonstrated strong similarities (7). The outbreak of 2007–2018 and a lesser one in 2014 coincided with irruptions of common voles, *M. arvalis* (89), in Castilla y Leon. US, Soviet, and Swedish workers have long recognized harvest activities and rodent irruptions as major risks for tularemia (68, 77, 115). The role of animals that do not die of tularemia as chronic shedders that continue to contaminate the environment remains poorly

studied. Voles, for example, apparently develop a chronic nephritis and bacteruria (12), a finding that was confirmed for voles that were orally infected (108).

7. GENERAL COMMENTS ON TULAREMIA VECTOR STUDIES

Rodents are exquisitely sensitive to infection by *F. tularensis*, dying within the week of a sepsis that is characteristically identified by gross lesions on necropsy (31). Tularemic pathology includes prominent, often caseous lymphadenopathy and readily visible pinpoint white spots on the surface of the liver and spleen, representing necrotic foci with masses of bacteria. Thus, early transmission studies either allowed potential vectors to feed on a rodent or homogenized vectors and inoculated the homogenates into rodents, then waited for a characteristic rapid death and easily scored gross pathology. Good confidence can be placed in these assays even today, in the times of ultrasensitive molecular diagnostic methods. Definitive assays rely on recovery of the agent by cultivation or its safer surrogate (necropsy of tularemic animals is hazardous and propagation in vitro is even more so), evidence of bacterial DNA by polymerase chain reaction (PCR). PCR, however, must be done with stringent contamination control to prevent false positives and usually fails to discriminate viable bacteria from DNA remnants. Strong evidence for viability is thus provided by results from animal inoculation or cultivation, but PCR or immunofluorescence methods may have other interpretations. Accordingly, studies from the older literature can be considered to have used sound diagnostic assays and should not be discounted due to their age. However, older experimental studies used challenge strains that were uncharacterized, and it is even unclear whether they were Type A or B; exceptions are Davis (29) and Hopla (64), who used a strain then called Sm, which is the current Type A standard, Schu.

There is much literature on field surveys for *F. tularensis* in vector arthropods, but other than the definitive establishment of the presence of a transmission cycle, sometimes very little can be concluded from such data. Removal of hematophagous arthropods from a host rarely allows for any conclusive evidence of vector competence because infection may be present in freshly ingested blood, as opposed to having been retained from a previous blood meal. Nest parasites such as mites, fleas, or lice must frequently feed on a host, and many do not survive long without a blood meal. The critical question for the enzootic cycle is whether bacteria remain viable within such arthropods, live or dead, in the absence of the host, implying that a new host acquiring them (or eating them or becoming exposed to their products, such as flea dirt) could become infected. PCR is almost exclusively used today to detect microbial agents during vector surveys, but the specificity of the primer sets used for detecting *F. tularensis* varies. The great diversity of *Francisella* spp. that is now known may confound interpretation of some of the prevalences previously reported by tick surveys, particularly those that used PCR primers that have not been directly tested against newly recognized *Francisella* spp. (24).

7.1. Fleas

During the first investigations of the biology of *F. tularensis* by McCoy & Chapin (97), fleas were implicated as maintenance vectors. Both *Ceratophyllus acutus* (*Diamanus montanus*) and *Ceratophyllus fasciatus* were experimentally infected by feeding on tularemic guinea pigs and ground squirrels, but infectivity for more than a day or two after feeding was not tested. In addition, although 100 fleas removed from a guinea pig that had died of tularemia were placed in a clean cage with a healthy squirrel, resulting in death of the squirrel, it was not clear whether the squirrel acquired infection by flea bite or by ingesting groomed fleas. Of course, either mode may be effective in enzootic maintenance. Experimental evidence suggests that fleas (*Xenopsylla cheopis* and *D. montanus*) may acquire bacteria from infected mice and retain viable infection for more than a month (128)

but not transmit it by feeding. Earlier studies (111) with three additional flea species, including *Pulex irritans*, demonstrated survival for only a day and no transmission. Larval fleas fed cultivated *F. tularensis* could retain infection for no more than three days and did not become infected by feeding on dried blood that had been spiked with culture (62). Surveys of diverse flea species demonstrated natural infection (58, 152; for summary, see 135), but early surveys for plague in the western United States, using the technique of inoculating flea homogenates into rodents, rarely found *F. tularensis* (5), suggesting that fleas were a very minor contributor to the maintenance of this infection, at least in the American West. Thus, the published information on the role of fleas as enzootic vectors remains inconclusive.

7.2. Lice

The rabbit louse (*Haemodipsus ventricosus*) transmitted *F. tularensis* to experimental rabbits, but transfer of lice from an infected animal to an uninfected animal needed to occur within three hours or infection generally failed (44). However, a few rabbits became infected with lice that had been held for 2–3 days. Francis was very careful to exclude the possibility that infection was due to contamination with secretions or excreta of dying rabbits by placing hair with lice from the dying donor onto naïve rabbits and placing the rabbit within newly cleansed trash cans. Briefer but similar experiments by Francis' team using *Polyplax serratus* (46) also demonstrated transmission even a week after lice had been removed from the infected hosts. Francis was careful not to state that infection was due to bites by the lice (recognizing that mice will groom and eat lice) and simply pointed out that mouse infestation led to transmission under conditions that excluded a healthy mouse's contact with the excreta of infected mice. Price (126, 127) experimentally infected human body lice (*Pediculus humanus corporis*) by feeding them on rabbits that had intravenously received a large dose of cultivated *F. tularensis* just before serving as host, as well as a cohort infected by intracoelomic inoculation. Serial sections of infected lice were examined to determine the course of infection over time. Interestingly, there was relatively little multiplication of bacteria in those infected by feeding (ingestion of 6 log cfu and measurements of not much more than 6 log cfu days thereafter). Those inoculated with 3 log cfu demonstrated 3 log multiplication within four days, with quick progression to mortality. Price suggested that when bacteria remained confined to the gut, lice were more likely to survive, but that nutritional factors in the hemolymph allowed for rapid multiplication and toxicosis, quickly leading to louse death. Given the host specificity of most lice, the fact that new hosts become infested only by very close contact (lice do not persist in fomites), and the very short life of lice in the absence of a host, lice can at most help to amplify infection during an epizootic but would not maintain the agent once all the hosts for that louse species died. Of course, the lice themselves would soon become locally extinct if their hosts were not present.

7.3. Bedbugs

Francis & Lake (45) fed bedbugs (*Cimex lectularius*) on infected mice and guinea pigs and confirmed three modes of transmission: (*a*) by interrupted feeding (the bug was removed from infected animal before repletion and allowed to reinfest a naïve animal), (*b*) by bite after as many as 71 days (infection by bite was ensured by allowing bugs to feed on mouse tails), and (*c*) by allowing mice to eat bugs infected as many as 100 days previously. The possible role of cimicids or triatomines as potential interepizootic hosts has not been explored.

7.4. Flies

Tularemia was first described as a disease by Francis during investigation of a deer fly–transmitted outbreak in Utah. Using field-collected *Chrysops discalis*, Francis & Mayne (47) successfully

transmitted infection by the bite of flies that had fed for short durations (interrupted feeding) on an infected guinea pig or rabbit and, hours to days later, were fed on uninfected guinea pigs, which died of typical tularemia. His team subsequently assayed flies that had fed on infected guinea pigs on a daily basis, injecting fly homogenates into uninfected guinea pigs. "Up to 5 days the flies remained constantly infected" (47, p. 1745), but Francis & Mayne argued that infection tended to become lost by day 10 after infection and thus there was likely no replication. Thus, flies were considered to be mechanical vectors. Pavlovsky (116) clearly recognized the vector role of tabanid flies and in fact equated their role in tularemia maintenance with their role in that for anthrax: Flies would aggregate on moribund animals and spread the infection by contaminated mouthparts and interrupted feeding on new hosts. Soviet workers incriminated numerous species in the genera *Tabanus*, *Chrysops*, *Stomoxys*, and *Haematopota* in the spread of tularemia infection (135, cited in 49). Interestingly, reports of fly-transmitted tularemia are very rare in places other than the American west, and indeed, Jellison (72) went so far as to state that the only species of any importance as a vector was *C. discalis*. From our own work, *F. tularensis* DNA may rarely be found in *Chrysops vittatus* in our Martha's Vineyard site, and we know of an ulceroglandular case acquired from a deer fly bite on Nantucket (T.J. Lepore, S.R. Telford, and H.K. Goethert, unpublished data). It is likely that fly-transmitted infection may be found in most endemic sites, but human ulceroglandular cases are automatically considered to be a result of tick bite in the absence of dermal exposure to contaminated materials.

7.5. Mosquitoes

Philip & Parker (122) first examined the possible transmission of *F. tularensis* by bite, using local species of *Aedes* as well as *Aedes aegypti*. Mosquitoes were infected by feeding on tularemic guinea pigs. No mosquito transmitted *F. tularensis* by bite during a second blood meal, but a small proportion did so after interrupted feeding (this was interpreted as mechanical transmission). Swatting infected mosquitoes onto the skin of guinea pigs also transmitted infection. The excreta deposited after an infectious blood meal was infectious when inoculated into guinea pigs. Philip & Parker also noted that *F. tularensis* survived in dead mosquitoes for at least four days and speculated that this could serve as a means to contaminate bodies of water, reminiscent of Manson's classic findings with filariasis (25). Soviet workers regarded mosquitoes as tularemia vectors but of minor importance to the maintenance of natural foci compared to ticks and tabanids (116).

Because inoculation ulcers are found most frequently on the trunk, neck, and ears of case patients, tularemia appears to mainly be mosquito transmitted in Sweden (36). *F. tularensis* has been isolated from mosquitoes there (105), and mosquito larvae collected from endemic sites that were allowed to develop and emerge as adults in the laboratory contained *F. tularensis* DNA (88). Out of 791 host-seeking mosquitoes collected from endemic Swedish sites, 18 were positive for *F. tularensis holarctica* DNA (146); infection was detected in *Culex*, *Aedes*, *Anopheles*, and *Coquilletidia* spp. In a definitive experiment, second-instar *A. aegypti* larvae were exposed to 7 log cfu of Type B, then washed and allowed to develop to adults. One-quarter of the adult mosquitoes contained *F. tularensis* DNA, and of those that did, the homogenates from one-third infected mice when intraperitoneally inoculated, demonstrating that virulent bacteria were transstadially passed (9). Larval *Culex quinquefasciatus* readily fed on biofilms of *F. tularensis holarctica*, but their pupation was delayed, and emergent adult mosquitoes were smaller than those feeding on the control dog biscuit slurry (91). Additional studies are needed on the vectorial capacity of mosquitoes for *F. tularensis*, particularly to clarify the mode of transmission; to date, studies have generally failed to transmit by bite, and the leading hypothesis is that people become infected by swatting a feeding mosquito and contaminating their skin. Short-lived antibodies to mosquito salivary (86) or gut proteins might

be sought in tularemia patients to further confirm mosquito transmission. Although experimental mosquito-related transmission rates appear to be small, the sheer abundance of mosquitoes in nature would compensate for low-probability events. Given the evidence that humans acquire infection from mosquitoes, it is a certainty that other animals in enzootic sites do as well.

The role of mosquitoes may be specific to some natural foci. Over 9,000 mosquitoes were screened with negative results from a site in the Czech Republic, and as many of 2% of host-seeking ticks that were concurrently collected yielded *F. tularensis* isolates (69).

7.6. Mites

Mesostigmatid mites (*Hirstionyssus*, *Laelaps*, *Eulaelaps*, *Haemolaelaps*) were found by Francis & Lake (46) and Soviet workers cited in Reference 49 to be naturally infected by *F. tularensis*. Painstaking studies done by Cluff Hopla for his doctoral dissertation (63) demonstrated the likely role of hematophagous mites in maintaining *F. tularensis*. Using *Ornithonyssus bacoti*, the tropical rat mite, Hopla determined that protonymphs acquired infection from septic mice and that bacteria were transstadially passed into the nonfeeding deutonymph and could be transmitted by the adult mite to clean mice. Clear evidence of transovarial transmission was also provided, although only for approximately one-fifth of the adult mites feeding on infected mice. Transmission was not by bite, but rather required the mice to groom and eat the mites. Assays for infection comprised both mouse inoculation and cultivation on glucose blood agar. Cultivation allowed for estimation of bacterial burdens, which, on average, were greater than 6 log cfu per mite even after prolonged fasting. Given the great diversity of blood-feeding mites, some effort should be made to better describe the vector–pathogen associations in the laboratory and in nature.

7.7. Soft Ticks

Davis (29) determined that *Ornithodoros turicata* and *Ornithodoros parkeri* could remain infected by *F. tularensis* for over 600 days but failed to transmit infection by bite. Detailed studies of infected *Ornithodoros moubata*, *O. parkeri*, *Ornithodoros hermsi*, and *O. turicata*, including infection with a known Type A strain (Schu), found viable bacteria for 450 days and demonstrated viable *F. tularensis* in rectal secretions and coxal fluid, as well as transmission by bite (20). Contamination by coxal fluid appeared to be the most likely means of transmission, as *O. hermsi*, which does not secrete liquid coxal fluid during feeding, was the least competent vector. No evidence of infection-related mortality was reported, despite numerous bacteria colonizing virtually every tick tissue. The fact that soft ticks can maintain viable infection for such extended durations suggests that they may help maintain a tularemia natural focus where they are endemic (the southern, southcentral, and western United States and central Asia).

7.8. Hard Ticks

The American ixodid ticks *D. variabilis*, *D. andersoni*, *Dermacentor parumapertus*, *Amblyomma americanum*, and *Ixodes scapularis* are competent vectors for *F. tularensis*, as demonstrated by laboratory studies or by case reports implicating the tick species (2, 11, 64–66, 114, 123, 139). Soviet workers, particularly Petrov & Dunaeva (121) and Petrov (119, 120) (cited in Reference 10), demonstrated intense multiplication and survival of the agent for as long as 700 days within *D. reticulatus*. Balashov (10, p. 352) stated that ticks are "the most effective natural vector and reservoir" given the many demonstrations of transmission by feeding, intensive multiplication of the bacterium, long survival within ticks with no loss of bacterial viability or virulence, and frequent detection

of infection in surveys of host-seeking ticks. The multiyear life cycles of most ixodid ticks make them a logical candidate for long-term persistence of *F. tularensis* natural foci.

The competence of *D. variabilis* for *F. tularensis* was measured in two exemplary modern studies (130, 131), which set the standard for any future work. Realistic doses (100 cfu) were used to infect mice. Two Type A (A1b, A2) strains and one Type B strain were compared. Experimental ticks were from a longstanding laboratory colony with precise provenance and known to be free of any specific pathogen. Uninfected cohorts were generated from the same tick batches for comparison with the infected ones. Sample sizes were such that statistical comparisons were adequately powered. Assays for infection were definitive: cultivation of the agent from individual ticks. The methods were presented in such detail that the studies could be replicated by other laboratories. The experiments demonstrated that A2 strains caused nymphs to die quickly, but not adult ticks. Infection by any of three strains affected nymphs in some way, by reducing body size, attachment, or feeding success. The A1b strain was not transmitted to mice by infected nymphs, and transmission was poor for the A2 and B strains (8–12% success). Mice could become infected by eating the infesting infected nymphs. Although adult *D. variabilis* never feed on rodents, this host restriction was overcome by confining ticks to capsules on mice; of those female ticks that fed and were shown to have contained infection, transmission occurred 58–89% of the time. Accordingly, the competence of one characterized strain of *D. variabilis* differed depending on the infecting *F. tularensis* type, but all three types were transmitted by the bite of adult ticks, and two were transmitted by the bite of nymphs. *F. tularensis* was inferred to have been transmitted within one day of attachment by infected ticks based on the observation that some mice were diseased within four days of repletion; this was calculated using a known prepatent period of three days and a feeding duration of seven days for female dog ticks.

Many surveys of host-seeking ticks have been published. As with many other tick-borne infections, there is no standardization of assays; with PCR assays, specificity should be demonstrated to prevent detection of FLEs or environmental *Francisella* spp. Sample sizes are often such that confidence intervals around the estimated prevalence are very wide. Thus, it can be difficult to compare published prevalence estimates. Two very detailed surveys for *F. tularensis* infection in host-seeking ticks provide examples of what would be most informative. Nearly 8,000 *D. reticulatus* were sampled from a natural focus in the Czech Republic during 1995–2013 (70); 64 *F. tularensis* isolates were recovered by mouse inoculation, for a minimum infection rate of 0.83%. The bacteriological gold standard is isolation of the agent. Goethert et al.'s (52, 53; H.K. Goethert and S.R. Telford, unpublished data) observations of the Martha's Vineyard natural focus during 2001–2011 comprise PCR detection in ticks (median of 1,572 host-seeking adult *D. variabilis* tested each year) with a median annual prevalence of approximately 3.1% (range 0–5.2). Two gene targets were used (*fopA* for the initial screen and *tul4* for a confirmatory assay), and a large proportion of those samples with specific DNA were genotyped (52, 53). Detection of genetic material, however, does not establish viability.

7.8.1. Transovarial transmission. The literature contains contradictions with respect to the inheritance of *F. tularensis* by ticks. Three reports (64, 65, 113) demonstrated inherited infection that would pass transstadially to the adult when larvae and nymphs were fed on uninfected hosts. Parker & Spencer (113) definitively demonstrated inheritance of the progeny of 2 of 15 female *D. andersoni* feeding on infected rabbits; the evidence comprised transmission by bite from larvae or nymphs. Another 6 of the 15 were suggestive of inheritance, but typical infection (death of the host) was not demonstrable; infection was inferred only by transfer of splenic material from the rabbits fed on by the progeny to uninfected animals. Calhoun & Alford (23) found infection of host-seeking *A. americanum* larvae when animals were inoculated with homogenate pools. In

contrast, Soviet workers suggested that any demonstration of transovarial infection was due to contamination of enclosed larvae by secretions of an infected female tick (120) and dismissed the possible contribution of inheritance by the tick as a means of *F. tularensis* perpetuation (116). Bell (11) failed to demonstrate inheritance in *D. variabilis*. More recent analyses by Genchi et al. (50) did not find transovarial transmission from female *D. reticulatus* and *I. ricinus*, with bacteria appearing to die within previtellogenic oocytes. They noted that 17–30% of all engorging female ticks died or did not lay eggs. It may be that the efficiency of the process may vary among strains of *F. tularensis* and could even be associated with tick genetic background (coadaptation of vector and pathogen).

7.8.2. Paradox of fitness effects due to infection. Philip & Jellison (123) first reported that ticks infected by the agent of tularemia were likely to die or fail to oviposit. A recent thorough analysis (130) clearly documents that infection of *D. variabilis* by Type A diminishes nymphal survival; infected nymphs were generally smaller and took twice as long to feed to repletion as uninfected nymphs. Interestingly, these negative fitness effects were not seen with adult dog ticks (131). Type B–infected nymphal *D. marginatus* or *D. variabilis* die more rapidly than do uninfected ticks (130), and in fact, only 2% of all *D. marginatus* feeding as larvae on infected animals developed to the adult stage (119, 120). The faster that host-seeking adult *D. variabilis* from the Martha's Vineyard site died in captivity, the more likely they were to contain *F. tularensis* DNA (56). Diminished longevity of ticks would impact reproduction, which implies that vector competence for Type A should be selected against; however, at least in the Martha's Vineyard natural focus, dog ticks containing *F. tularensis* DNA have been found every year for more than 15 years (H.K. Goethert and S.R. Telford, unpublished data). Like *Rickettsia rickettsii*, which is also generally a lethal infection for *D. andersoni* (102), the continued demonstration of infected tick vectors in the field suggests that, despite *F. tularensis* negatively affecting fitness, there is some as-yet-unidentified compensatory effect that maintains the enzootic vector–pathogen relationship. For example, *F. tularensis* genotypes or lineages might be specifically coadapted to local tick populations, and the elegant experiments of Reese et al. (130, 131) should be repeated with diverse *D. variabilis* colonies and *A. americanum*, the two main zoonotic vectors for Type A.

7.8.3. Allergic klendusity. During vector competence studies of *D. variabilis*, Bell (11) noted that a rabbit fed upon by *F. tularensis*–infected ticks failed to become infected. That rabbit had previously been fed on by uninfected ticks, and he speculated that sensitization interfered with transmission. In a subsequent experiment, it was found that rabbits previously fed on by *D. andersoni* (usually larval infestation) were half as likely to be infected by *F. tularensis*–infected ticks (13). Bell designated this allergic klendusity (disease-escaping ability) and theorized that it was due to antitick immunity, referring to Trager's classic experiments (150). This interference was manifested at the portal of entry, working for the two possible modes of transmission, e.g., by bite of an infectious tick or by contamination of the bite site with tick feces containing *F. tularensis* while the tick is feeding. Bell also suggested that such a mechanism might serve to limit natural epizootics. This paper serves as the observation that is the basis for current efforts in antitick vaccines and current understandings of their promise for protecting against tick-borne infection.

7.9. Diverse Experimental Arthropods

Advances in our understanding and development of arthropod models for innate immunity have led to some interesting infection models of *F. tularensis*. *Drosophila* (101, 153), dubia roaches (34), and caterpillars [*Galleria mellonella* (6, 148); *Bombyx mori* (143)] have been experimentally infected

by inoculation of cultivated *F. tularensis*, typically with dose-dependent mortality that was more pronounced at mammalian temperatures than arthropod (lower) temperatures, although *B. mori* survived for at least a week. Antimicrobial peptides such as those in the *imd/relish* pathway appear to be activated and prevent overwhelming sepsis; flies with that pathway knocked out succumb rapidly to infection (101). Melanization was inhibited in *B. mori* inoculated with live *F. tularensis* but not with dead bacteria, suggesting that some active bacterial response or secretion inhibits innate immunity. *F. tularensis* mutants with critical virulence genes (as determined for mammalian infection) also survived longer, suggesting that at least some known virulence factors apply to both mammals and arthropods. The new experimental hosts confirm what has been known since Francis' first investigations: that *F. tularensis* has an extremely wide arthropod host range, including acarines, dipterans, lepidopterans, hemipterans, anoplurans, and siphonapterans, and thus that the infection should not be considered to be limited to solely vector species.

7.10. Aquatic Invertebrates

Shrimp or snails could retain viable organisms for 20 days (99). Soviet scientists were the first to describe invertebrates contributing to *F. tularensis* survival within water: Pavlovsky (116, p. 106) states that the "tularemia microbe…may be found in the bodies of… mollusks, crabs and crayfish, water bug larvae…." Crayfish have been associated with human infection, and *F. tularensis* apparently infects them (4), suggesting that additional surveys of aquatic invertebrates using modern methods are warranted in known natural foci.

8. ARE VECTORS REALLY REQUIRED FOR MAINTENANCE?

Although 10–100 cfu of Type A are sufficient to produce a lethal infection in most mice with most *F. tularensis* strains when delivered by aerosol (26), and 1,000 cfu is a typical LD50 for parenteral inoculation (147), LD50 is 6 log cfu for oral infection (gavage). Mice survived oral infection with 4 log cfu (82), suggesting that eating materials contaminated with *F. tularensis* (via consuming excreta on food, grooming ectoparasites, or cannibalizing moribund animals) could maintain transmission during epizootics and perhaps even over the long term within natural foci [e.g., by eating infected starved bed bugs or soft ticks (45, 63)]. In the laboratory, Type A and B infections may be transmitted via cannibalism of cagemates that succumb to tularemia (108, 110). Cannibalism may play a role in the enzootic cycle as well: Partial immunity due to ingestion of sublethal doses of *F. tularensis* may allow for survival of a rodent that is shedding bacteria (12). Such immunity suggests a means of regulating the duration of epizootics and developing a new endemic focus.

Water-borne tularemia was first described by Karpoff & Antonoff (77) in their description of an outbreak related to drinking unboiled brook water; 43 hay harvesters had evidence for hyperemic oral mucosa, tonsils, or conjunctiva. Brook water readily yielded isolates of *F. tularensis* when inoculated into guinea pigs. In the United States, cold waters contaminated by muskrat and beaver repeatedly yielded isolates of *F. tularensis* (74, 115). Infected carcasses contaminated water and, when stored in the cold, provided tissues that infected animals after two weeks; naturally contaminated mud remained infectious for as long as 10 weeks (115). Type B DNA has been found in water and sediment in Swedish endemic sites, even in years with no zoonotic transmission (19). However, Type B held in lake water for 120 days failed to kill mice at a dose 10 times the typical LD50 for that Type B strain (147), implying a loss of virulence that was not protected by the presence of high nutrient levels or free-living protozoa. About half of rodents immersed in contaminated water became infected with exposure to as few as 100–1,000 cfu/ml (116), which may seem like heavy contamination, but the spleen alone of a mouse dying of tularemia may have 10

log cfu (100); Pavlovsky (116) estimates that a single dead rodent could infect 500,000 liters of water. Environmental persistence may depend on continual contamination of the environment by infectious carcasses (115, 116, 125). The persistence of *F. tularensis* in nonaquatic environments has received little attention; the bacterium has been lyophilized in a protein matrix and remained viable for four years in ampules stored on a desktop (98), suggesting the possibility for long-term persistence in natural foci. We speculate that a key factor in the endemic pneumonic tularemia focus of Martha's Vineyard, in which landscapers comprise a major risk group (38), is the exposure of soils there to oceanic salt sprays (16) that would promote the viability of contaminating bacteria.

Bacterial endobiosis with free-living amoebae or other cyst-forming protozoa could serve to contaminate the environment with viable bacteria for a longer duration than if the bacteria were present in an extracellular or naked form. Although *F. tularensis* is said to be environmentally resistant, the naked bacteria are fragile and do not survive in the laboratory for more than two to three weeks in spring water or saline (16); in another study, though, Type B survived 70 days in tap water at 8°C (39). *F. tularensis* infects amoebas and ciliates in the laboratory (1, 14, 35) and can enter a viable but nonculturable state (39). Viable but nonculturable states might imply the possibility of long-term persistence in the environment, with reversion to replicating, infectious bacteria.

9. FUTURE DIRECTIONS FOR RESEARCH ON TULAREMIA ECOLOGY

Some open questions will be challenging to answer given the difficulty in finding longstanding natural foci and the inability to experimentally manipulate conditions in the field. How long does infection persist in carcasses or remnants thereof when placed within suitable substrate? Does survival vary according to site physiography or even more specific microhabitat requirements (salinity, soil acidity, or other chemical attributes)? The classification of *F. tularensis* as a US federal Select Agent (meaning that it is of interest to biodefense, and thus that any possession or manipulation is highly regulated by the government) (**https://www.selectagents.gov/regulations.html**) makes even the most basic of deliberate field experiments impossible to undertake in the United States. It would be virtually impossible to bury fresh infected mouse carcasses or infected ticks in a field site (i.e., using a site that was known to be endemic and a *F. tularensis* strain derived from that site) and sample them periodically to determine the duration of bacterial viability because of fears that they might be hijacked for nefarious purposes.

In the United States, much attention has been focused on ticks; can Type A be enzootic where there are few ticks, as demonstrated for Type B in Sweden? Can a natural focus disappear with continued application of acaricides to reservoirs or to the environment? Long years of antitularemia campaigns by the Soviets rarely attacked just the ticks, instead relying on human vaccination, rodent elimination, and general sanitation (125); however, these efforts failed to eliminate natural foci.

In this review, we argue that our knowledge of the ecology of tularemia is incomplete mainly because past studies have focused on the zoonotic condition, as opposed to identifying the requirements for maintenance. Of course, human exposure (the subject of epidemiology) may provide clues to the mode of perpetuation (ecology), but this is not axiomatic. Zoonotic infections may exist in sites with no implied human risk in the absence of an effective epidemiological bridge. Even the very concept that *F. tularensis* is an obligate vector-borne infection remains to be proven: Its ecology may be more like that of *Coxiella burnetii*, the agent of Q fever (with diverse modes of perpetuation), than that of *Rickettsia* spp. The former is environmentally opportunistic; there are obligate and specific vector–pathogen associations for the latter.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Our work on tularemia was supported by National Institutes of Health (NIH) grants R21AI 53411 and R01 AI064218. We are currently supported by NIH grants R01 AI 130105 and R01 AI 137424, by the Rainwater Foundation, and by a gift from Catherine C. Lastavica. This is a contribution of the New England Regional Biosafety Laboratory.

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