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Survival of *Francisella tularensis* Type A in brackish-water

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Abstract

Martha's Vineyard (MV), Massachusetts has been the location of two outbreaks of pneumonic tularemia; landscaping activities have been associated with risk, suggesting environmental inhalation exposure. We determined whether salinity or other components of brackish-water present in a location with endemic tularemia may prolong survival of *F. tularensis*. In addition, we demonstrate for the first time that *F. tularensis* Type A appears similar to Type B with respect to environmental stability. The results of this study suggest an explanation for why MV is the site of pneumonic tularemia transmission as opposed to sites in the southcentral USA, where tularemia is more commonly reported: Bacteria may be more prone to surviving in salt-influenced soil or moisture in the island setting.

Keywords

Tularemia; Environmental stability; Microcosm; Type A; Martha's Vineyard

The occurrence of pneumonic tularemia on the island of Martha's Vineyard, Massachusetts is highly suggestive of an unusual relationship between the ecology of the island and the human activity on this island. From 2000 to 2006, 59 cases of tularemia due to *Francisella tularensis* subsp. *tularensis* (Type A) were reported, of which nearly 2/3 were pneumonic in clinical presentation (Matyas et al. 2007). A case-control study conducted by the Center for Disease Control and Prevention and the Massachusetts Department of Public Health established that landscaping activities on the island increase the risk for tularemia (OR, 6.7; $P = 0.04$), suggestive of an aerosolization of *F. tularensis*-contaminated environmental material while undertaking these activities (Feldman et al. 2001, 2003). Field investigations, however, have failed to identify the fomites that serve as an aerosol risk Feldman et al. (2001).

Although *F. tularensis* subsp. *holarctica* (Type B) is well known to contaminate and survive in freshwater and has been identified in environmental matrices contaminated by infected animal sources (Dahlstrand et al. 1971; Jellison et al. 1942; Parker et al. 1951; Pollitzer et al. 1967; Stewart 1996; Syrjala et al. 1985), *F. tularensis* Type A has yet to be isolated from such sources. Indeed, we are unaware of peer-reviewed evidence that Type A may persist for prolonged periods in the environment; reviews of the biology of tularemia conflate what is known for Type B with evidence for Type A to have such capacity. There are approximately 249 hectares of freshwater or mostly freshwater ponds on Martha's Vineyard, and 27 coastal/salt ponds that cover nearly 3645 hectares on this island of 22, 550 hectares

(<http://www.mvcommission.org/planning/ponds.html>); surface water comprises 17% of the total land area. Exposure histories provided by tularemia cases include activities around the brackish-water-influenced southern portions of the island (Matyas et al. 2007). It may be that salinity, or some other component in these water sources, enhances persistence of viable *F. tularensis* in environmental media and thereby contributes to risk. Accordingly, we determined whether the survival of a Martha's Vineyard *F. tularensis* Type A (SSTR9 10 7) isolate was enhanced in brackish-water samples collected from our Chilmark field site on Martha's Vineyard (Goethert et al. 2004).

We compared the duration of in vitro growth and survival of *F. tularensis* Type A (SSTR9 10 7), which is the most common haplotype found on Martha's Vineyard (Goethert et al. 2004) with that of Type B LVS (Live Vaccine Strain, LVS; BEIR #NR-14), and *F. novicida* U112 (BEIR #NR-13). Culturability (as opposed to viability) of suspensions of these bacteria in freshwater and brackish-water microcosms was assessed by measuring bacterial colony-forming unit counts (CFUC) over time. All cultures were performed using cystine heart agar blood (CHAB) supplemented with 8% rabbit blood and antibiotics (Remel, Lenexa, Kansas). Freshwater and brackish-water samples were collected from Fulling Mill Brook and Squibnocket Pond, Martha's Vineyard, respectively, which are representative of surface water on that island. Water samples were tested for evidence of *Francisella* spp. DNA by PCR prior to use; none was found. The water and normal saline (0.85% NaCl) used in the experiments was filter-sterilized using 0.1- μ m pore-sized filters (Millipore) and stored at 4°C until used.

Briefly, a loopful of bacteria was carefully removed from CHAB plates and resuspended in sterile normal saline to wash the cells prior to inoculation. The bacteria were pelleted by centrifugation, the supernatant removed, and the pellet was resuspended in normal saline. Optical densities at 590 nm (OD₅₉₀) were measured for a 1:10 dilution of bacterial suspension in 10% formol saline to standardize the inocula for each strain to obtain an OD₅₉₀ = 0.03. Working dilutions of each bacterial strain were made (1:100) in brackish-water, freshwater, and normal saline; 1.6 mL of each strain suspension was aliquoted into 2.0-mL screw top, polypropylene tubes (Axygen, Union City, California) and stored at room temperature (approximately 21°C). Serial dilutions of each strain suspension were made in the respective media in which the suspension was composed (i.e. brackish-water, freshwater or saline), and 10 μ l of three dilutions was plated on CHAB in triplicate to assess growth or decay. All cultures were grown at 37°C until growth was easily observed. Suspensions were inoculated on CHAB at days 0, 2, 5, 7, 10, 14, 18, 21, 28; and day 34 if robust growth was observed on day 28. Three separate replicates were performed for the experiment. All graphs and statistical analyses (Kruskal–Wallis one-way ANOVA, Mann–Whitney *U* test) were performed using GraphPad Prism (v. 3) on the means of the normalized CFUC values obtained from the triplicate counts of each independent experiment. CFUC values were normalized to obtain approximately the same starting value across the three independent experiments for each media type and strain tested. Mean CFUC values for each time point were multiplied by the factor that would bring the day 0 values closest to the most concentrated mean CFUC day 0 value.

Initial mean CFUC values from working saline suspensions used to inoculate the water media ranged $\sim 1 \times 10^8$ – 2×10^9 CFU/mL; the exception is one experiment for *F. novicida* with the starting mean value of $\sim 2 \times 10^7$ CFU/mL. For all experiments, there was no significant increase in CFUC in saline, fresh and brackish-water media for all strains tested indicating no exponential growth in the absence of added nutrients. A rapid decrease in colony counts was observed for all strains in freshwater, with no CFU observed after 7–10 days (Fig. 1a). Colony counts for all strains declined much less rapidly in brackish-water suspensions, with Type B LVS persisting longer than the Type A strain by at least 7 days

(Fig. 1b). Type B LVS performed poorly in saline relative to the Type A strain with a more rapid decline in CFUC at day 21 (Fig. 1c). Previous experiments with an attenuated Type A strain (B38), a known fastidious *F. tularensis* strain (Payne and Morton 1992, personal observation), showed culturability to at least 14 days from brackish-water, and typically no growth after 1 day in freshwater (data not shown). Thus, brackish-water is superior to freshwater in sustaining *F. tularensis*, and Type A is culturable from brackish-water suspensions to a comparable time point to that of Type B (LVS).

There was no statistically significant difference between CFUC for the strains over the entire time period for which counts were obtained in all three media tested (Kruskal–Wallis one-way ANOVA, $P > 0.05$); nor did CFUC differ between the two strains over the entire time period that colonies were observed (Mann–Whitney U test, $P > 0.05$). Statistically significant differences emerged only when earlier time points were omitted from the analysis with differences noted among the three strains in freshwater media at day 7 ($P < 0.0001$), saline media at day 18 ($P < 0.0001$), and brackish-water media at day 34 ($P < 0.0001$).

Simple water analyses (Environmental Testing & Research Laboratories, Leominster, MA; University of Massachusetts Soil Testing Laboratory, Amherst, Massachusetts) indicate that the freshwater used in these experiments contains low levels of several macro- and micronutrients compared with brackish-water (Table 1). In addition, the brackish-water samples contained relatively great sulfur residues (168–219 mg/mL) with only 2–5 mg/L contained in the freshwater samples. The association between sulfur compound levels in environmental media and the survival of *F. tularensis* has been noted in early studies, including the seminal work by Parker et al. (1951) who found that naturally contaminated mud and sediments were high in sulfides Parker et al. (1951). The presence of sulfur-containing compounds in brackish-water may serve to enhance survival of *F. tularensis*, as sulfur-containing amino acids, cysteine or cystine, are usually required for the cultivation of *F. tularensis*. Additionally, early studies on factors that enhance *F. tularensis* growth in culture have noted that optimal growth can be obtained from 1% NaCl in culture media (Tamura and Gibby 1943). Because brackish-water and normal saline support the culturability of *F. tularensis* for a longer duration than that observed with freshwater, salinity may be an important factor for stability; however, it is likely that the presence of sulfur would enhance the effects of salinity.

Conventional culture methods are typically used in testing bacterial content and loads (CFU/mL), particularly when assessing water quality. Culture-independent methods, such as live/dead staining and RT-PCR, are sometimes used in microcosm experiments to account for viable but non-culturable (VNBC) populations and capture cell responses (Fey et al. 2004; Forsman et al. 2000; Gonzalez-Escalona et al. 2006; van Veen et al. 1997). However, the fate of VNBC populations of bacteria and whether they might serve as a risk for exposure is not clear. Resuscitation and retention of virulence of Type B LVS from VNBC states was demonstrated to be ineffective by mouse passage, and a reduction in virulence over time has been demonstrated for artificially contaminated water in which animal passage was used as a bioassay for recovery (Forsman et al. 2000; Mironchuk and Mazepa 2002). Bacterial growth in culture may closely approximate the minimum threshold of viability for which human or animal infection would be implied.

Pneumonic tularemia cases from Martha's Vineyard are believed to be due *F. tularensis* Type A, as demonstrated by characterization of clinical isolates and tick field surveys (Farlow et al. 2005; Goethert et al. 2004; Matyas et al. 2007). Most known infections with Type A are associated with tick bites or handling of infected rodents or rabbits (Centers for Disease Control and Prevention 2002; Farlow et al. 2005). The issue of whether Type A

behaves similarly to Type B within environmental media is critical for analyzing the environmental background that has contributed to the disproportionate number of pneumonic tularemia cases on Martha's Vineyard. While environmental microcosms in the laboratory setting are not exact replicas for natural conditions, they can serve the purpose of providing baseline ecological information.

The contribution of environmental factors, such as the presence of brackish-water, to the unique and protracted pneumonic tularemia outbreak on Martha's Vineyard remains to be fully described. If contaminated carcasses or excreta are deposited in salt-influenced media on Martha's Vineyard, bacterial survival may be prolonged enough to more frequently permit human exposure through landscaping activities there than in other areas of the United States where tularemia is endemic.

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Fig. 1. Normalized CFUC decay curves in freshwater (a), brackish-water (b), and saline (c). Type A (SSTR9 10-7) is shown with a solid square and short-dashed line; Type B LVS (LVS) is indicated by a solid triangle and long-dashed line; and *F. novicida* (Fn) is indicated by an upside-down solid triangle and a solid line. Error bars indicate standard error of the mean from the three experiments. X-axis scales differ between the three graphs to show detail of the decay curves for each of the media

Table 1

Water analysis is fresh and brackish-water used in microcosm experiments

Water type	pH	Conductivity (umhos/cm)	T. D. S. ^a (mg/L)	Sodium (mg/L)	Potassium (mg/L)	Calcium (mg/L)	Magnesium (mg/L)	Sulfate (mg/L)
Freshwater	7.4	90.0	54.0	10.0	1.5	2.8	1.9	21.1
Brackish-water	7.4	19,320	11.4	3603.5	131.2	131.4	440.5	1417.8

^aTotal dissolved solids