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Quantum of infection of *Francisella tularensis tularensis* in host-seeking *Dermacentor variabilis*

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Abstract

The American dog tick, *Dermacentor variabilis*, is fundamental to the perpetuation of *Francisella tularensis tularensis* on Martha's Vineyard, Massachusetts, U.S.A. Although infected ticks are relatively common on the island, human cases deriving from tick bite are rare. It may be that the quantum of bacteria within these naturally infected ticks is frequently too small to cause disease. Accordingly, we quantified the amount of *F.t. tularensis* bacteria in host-seeking ticks from the island. Contrary to our expectations, the majority of the ticks harbor large numbers of bacteria (median 3.3×10^8 genome equivalents/tick). Such a large quantum of infection might suggest that aerosolization of the ticks themselves might comprise the proximal determinant of risk for the inhalational tularemia that is common on Martha's Vineyard. However, the paradox of fewer ulceroglandular tularemia cases than would be expected given the abundance of potentially highly infectious ticks remains to be solved.

Keywords

Francisella; Tularemia; Dog tick; *Dermacentor*; Quantum of infection

Introduction

Martha's Vineyard, Massachusetts, has sustained a long-term tularemia epizootic since 2001, with nearly 100 confirmed cases of Type A tularemia due to infection by *Francisella tularensis tularensis* (Feldman et al., 2001; Matyas et al., 2007, and Massachusetts Department of Public Health, personal communication). We have identified the American dog tick, *Dermacentor variabilis*, as fundamental to the perpetuation of *F.t. tularensis* on this island (Goethert et al., 2004). As many as 2–5% of the ticks there contain DNA of *F.t. tularensis* (Goethert et al., 2004). Given a typical June density of 300 ticks per person-hour of sampling, it is possible that residents may encounter as many as 6 infected ticks in an hour of walking within certain sites. Dog ticks are well known to attach to humans and indeed are considered the main vectors of Rocky Mountain spotted fever in the eastern U.S. (McDade and Newhouse, 1986). Despite this great potential entomological inoculation rate, only 14% of the reported cases have been classified as ulceroglandular tularemia attributable to dog tick bites (Matyas et al., 2007). Our estimates of prevalence are based upon detection of *F.t. tularensis* DNA within tick hemolymph

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by polymerase chain reaction assays, which have analytic sensitivity to fewer than 10 bacteria. It may be that the scarcity of tick-transmitted tularemia on Martha's Vineyard is related to low bacterial burdens within ticks, which would limit the number of bacteria transmitted ("quantum of infection") via the tick saliva during attachment. Accordingly, we determined the quantum of infection of *F.t. tularensis* in host-seeking dog ticks by means of real-time quantitative PCR.

Materials and methods

Questing *D. variabilis* were collected from vegetation as part of our ongoing ecological studies of tularemia at our long-term field sites near Squibnocket and Katama Martha's Vineyard, Massachusetts, in 2005, 2006, and 2008 (Goethert et al., 2004; Goethert and Telford, 2009). Hemolymph from ticks was initially screened for evidence of *F.t. tularensis* DNA by PCR in pools as described previously (Goethert et al., 2004). Real-time quantitative PCR (qPCR) was then used to determine the number of genome equivalents (ge) of *F.t. tularensis* in individual tick samples. Whole-tick DNA extracts were used if a second hemolymph sample could not be obtained. DNA was extracted from homogenates of individual ticks using the DNeasy kit (Qiagen Inc.). Primers targeting the *tul4* gene were used as described previously (Christensen et al., 2006). A freshly harvested suspension of *F. t. holarctica* live vaccine strain (LVS, ATCCBEIR NR-14) was plated in duplicate on cysteine glucose blood agar to produce a stock suspension with a known colony forming units (cfu). This stock suspension was then boiled and used directly to make the standard curve for qPCR. As reported by Larsson et al. (2005), only one copy of the *tul4* gene occurs in the *F. tularensis* genome; so 1 cfu=1 ge. The 28S ribosomal DNA gene for *D. variabilis* was used as an internal standard to control for the amount of tick DNA in each sample. Primers targeting the gene (Dv28S3449f-AAG TGG GAG GTC ACG GGA TAC, Dv28S3578r-AAG CGT GGA CAA CTC AAT GGT) were designed using PrimerExpress 3.0 software (Applied Biosystems). Serial dilutions of a 200-bp synthetic oligonucleotide were used to create a standard curve. Each sample was corrected for the amount of tick 28S rDNA. The result was expressed as genome equivalents of *F. tularensis* per pg (ge/pg) of tick 28S rDNA. Because pg of 28S rDNA is not intuitively informative, we sought to normalize our results to reflect the amount of bacteria that would be present in an entire tick. To this end, we sought to determine how much 28S rDNA is present in an entire tick. Whole ticks (n=5) were individually homogenized in 50 ul PBS and the entire sample extracted for DNA (Qiagen). The amount of 28S rDNA was then determined for each sample, and the median was calculated. The calculated median was close in value to the average, indicating that 5 ticks were a sufficient and representative sample. All RT-PCR results (ge/pg) were then multiplied by the median (882 pg/tick) to yield an estimate of ge in the entire tick; ge/pg * 882 pg/tick = ge/tick.

Results

Quantitative real-time PCR was done on 133 individual *D. variabilis* that had tested positive for *F.t. tularensis* during the initial screening. The number of bacteria per questing adult ranged from 0 (under the level of detection for this PCR) to 3.26×10^{11} ge/tick, with a median of 3.29×10^8 ge/tick. The distribution of bacterial burdens is skewed to the right (Fig. 1). Most of the ticks harbored a large number of bacteria, that is, 50% of the ticks tested had 10^8 or 10^9 ge/tick, with very few (5%) harboring less than 10^3 ge/tick. There was no significant difference in the bacterial burden of ticks between sample types (hemolymph versus whole tick extracts) or among years (data not shown).

Discussion

Real-time quantitative PCR demonstrated a great quantum of infection in the majority of host-seeking dog ticks sampled from Martha's Vineyard. A report of transmission of *F. tularensis*

by *Amblyomma americanum* provided a similar distribution of bacterial burden (as determined by colony counts from tick homogenates), with a mean of 6.7×10^7 bacteria per nymph (Hopla, 1960). The *F. tularensis* results stand in contrast to those reported for other tick-borne agents. The quantum of infection for Lyme disease spirochetes in the northeastern populations of *I. scapularis* (Burkot et al., 1994; Brunet et al., 1995; Wang et al., 2003) is represented by a distribution with a long right-hand tail: Most of the ticks tested contained a low density of spirochetes, and the minority had large numbers of bacteria. The methods used in these studies ranged from direct counting of spirochetes to antigen-capture ELISA and real-time PCR. Although the methods are not directly comparable and the estimates of the absolute number of bacteria varied, the resulting distributions reported were similar among all the studies. A similar distribution was obtained for *I. persulcatus* ticks infected with tick-borne encephalitis virus (Korenberg et al., 1992). To our knowledge, ticks have not heretofore been described to naturally contain any other pathogen that attains such great density as what we and Hopla (1960) have found for *F. tularensis*.

The minimum inoculum necessary to transmit infection via tick bite has not been investigated for *F. tularensis*. Subspecies and strains of *F. tularensis* differ with respect to their capacity to cause disease, and particularly by mode of inoculation; for example, the live vaccine strain kills mice when even a single bacterium is intraperitoneally inoculated, but mice may survive when 10^5 cfu are delivered intradermally (Elkins et al., 1992). Even individual strains may differ in infectivity depending on the mode of cultivation that results in changing the nature of a surface polysaccharide capsule (Eigelsbach et al., 1951). If we assume, however, that the “highly infectious” nature of *F.t. tularensis*, estimated to be 1–10 individual bacteria in controlled inhalation experiments (Jones et al., 2005), is representative of all strains then it seems unlikely that bacterial burden is the limiting factor for tick transmission of this agent on Martha's Vineyard.

Our finding that ticks contain large numbers of bacteria poses an epidemiological paradox: Ulceroglandular tularemia should be commonly reported from Martha's Vineyard but is not. Other possible explanations for this paradox may relate to human behavior (e.g., people find large dog ticks easily and remove them before they effectively attach), viability, or transmissibility of the agent within ticks. Our assay relies solely on DNA amplification and therefore we do not know whether the bacteria that we detected are viable. Furthermore, we did not attempt to determine the distribution of bacteria within the tick tissues. Only live bacteria residing in the salivary glands would be immediately available for transmission via tick bite. The agent of tularemia also is known to enter a viable but non-cultivable (VBNC) and presumably non-infectious state in the environment (Forsman et al., 2000), and it is possible that tick-bound *F. tularensis* is similarly in a VBNC state. On the other hand, homogenates of ticks containing *F. tularensis* DNA are capable of infecting mice and causing disease when injected; and, the agent may be recovered from such homogenates by cultivation on CHAB (Hopla, 1960). However, the proportion of bacteria within a tick that is viable and infectious remains to be defined.

A unique phenomenon, reactivation, characterizes most tick-borne pathogens and modifies potential risk of transmission as measured by the entomological inoculation rate. It is not known whether the tick-bound *F. tularensis* requires a reactivation process. The phenomenon of reactivation was, in fact, first described for the dog tick-transmitted RMSF (Spencer and Parker, 1923). Homogenates of ticks failed to infect and kill guinea pigs unless the ticks had previously been feeding for at least 24 h. An electron-dense slime layer was ultrastructurally demonstrated on reactivated *R. rickettsii* (Hayes and Burgdorfer, 1982) but not on those from non-fed infected ticks, suggesting a physiological change was required for attaining infectivity. Reactivation is manifested as a delay in transmission such that if infected ticks are removed less than 24 h after attachment, a host fails to become infected. Such a “grace period” is characteristic for 3 of the

4 deer tick-transmitted infections (Lyme disease, babesiosis, and human granulocytic ehrlichiosis) (Piesman and Spielman, 1980; Piesman et al., 1987; Katavolos et al., 1998). Only deer tick virus (Ebel and Kramer, 2004) appears to be instantaneously transmitted upon attachment. Whether reactivation occurs and thus prompt removal of *F. tularensis*-infected dog ticks might prevent infection remains to be determined.

The great quantum of *F. tularensis* infection that appears to be present in host-seeking dog ticks on Martha's Vineyard may help to explain the frequency of reports of cases of primary (inhalational) tularemia there. The outbreak investigation for the index cases (Feldman et al., 2001) discussed the potential fomites that might serve as risk factors and included the suggestion of aerosolized ticks. Although the efficiency of homogenization of intact host-seeking dog tick adults by lawnmowers remains to be measured, the potential aerosol that might be generated would certainly contain enough of a quantum of infection to initiate pneumonic tularemia, assuming that all bacteria within a tick are viable and infectious.

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Figure 1.
The distribution of the burden of *Francisella tularensis tularensis* (ge/tick) in questing *Dermacentor variabilis* from Martha's Vineyard (Massachusetts, USA).