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Transmission of tularemia from a water source by transstadial maintenance in a mosquito vector

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Mosquitoes are thought to function as mechanical vectors of *Francisella tularensis* subspecies *holarctica* (*F. t. holarctica*) causing tularemia in humans. We investigated the clinical relevance of transstadially maintained *F. t. holarctica* in mosquitoes. *Aedes egypti* larvae exposed to a fully virulent *F. t. holarctica* strain for 24 hours, were allowed to develop into adults when they were individually homogenized. Approximately 24% of the homogenates tested positive for *F. t.* DNA in PCR. Mice injected with the mosquito homogenates acquired tularemia within 5 days. This novel finding demonstrates the possibility of transmission of bacteria by adult mosquitoes having acquired the pathogen from their aquatic larval habitats.

Vector-borne diseases are among the most complex of infections to predict and prevent. The intricate interactions among the ecological and behavioral traits of the pathogen, the vector, and susceptible hosts, need to be understood in order to prevent transmission that would otherwise lead to disease and burdens on society. Although mosquitoes transmit many pathogenic viruses and parasites, their ability to transmit bacterial diseases to humans is unknown. The transmission of tularemia by mosquito vectors has been suggested, but direct evidence for this specific mode of transmission is lacking[1–4].

Tularemia is a bacterial zoonotic disease of the northern hemisphere, endemic in certain geographical areas where it affects a wide range of mammals[5]. The causative agent, *Francisella tularensis*, is highly infective. It is included among Tier 1 agents on the US Select Agents list as a potential biological warfare agent[6]. The disease can be transmitted to humans by a number of different routes viz. inhalation of contaminated dust, ingestion of contaminated food or water, or via bites by infected vectors[5]. There are several clinical manifestations of tularemia, ulceroglandular being the most common form of disease associated with arthropod bites. Vectors associated with transmission of the two subspecies of *F. tularensis* that are of main clinical relevance in causing tularemia (subspecies *tularensis*, and subspecies *holarctica*) include hard ticks, deer flies, horse flies and mosquitoes[2]. The environmental sources of the pathogen, i.e. how the bacteria persist between outbreaks, are unknown in respect both of the two subspecies and the different infection routes. However, molecular methods have provided evidence that *F. tularensis* subspecies *holarctica* (*F. t. holarctica*) is frequently associated with natural water sources in endemic areas. Current data suggest that the bacteria can persist for prolonged periods in the environment, also between outbreaks[7–10].

In Sweden and Finland, clinical experience and epidemiological data indicate that mosquitoes are the main transmission route of human tularemia[11–17]. In fact, Finland and Sweden repeatedly report among the highest number of tularemia cases worldwide[18]. The mosquito has long been considered as a mechanical transporter of *F. t. holarctica*, it being assumed that it transfers the organism from infected to susceptible hosts during successive blood feeds[2]. In a field experiment, mosquito larvae were collected in an area of Sweden where tularemia is endemic. The larvae were brought to the laboratory and reared to adults. The adult mosquitoes tested positive for *F. tularensis* DNA so demonstrating that the bacterium may be transstadially transmitted by mosquitoes[3]. In addition, laboratory experiments performed on *Ae. aegypti* confirmed that mosquitoes exposed to the bacterium as larvae retain the bacterium internally, or at least its DNA, in all developmental stages through to the adult, and that the bacterium or its DNA is transmissible to blood vials during artificial blood feeding[4]. Attempts to culture the bacteria from such mosquitoes, and to experimentally transfer the infection to mice through mosquito bites, have so far been unsuccessful[2,4]. It is therefore not known if *F. t. holarctica* survives during the metamorphosis from larval to adult mosquitoes and if so, whether the bacterium retains virulence. Unraveling the mode of transmission would clarify the role of mosquitoes in tularemia outbreak dynamics as being mere amplifiers of

ongoing outbreaks, or as important factors in the initiation of outbreaks, i.e. as a bridge between the environmental reservoir of the bacterium and the susceptible hosts.

In the present study, we aimed to investigate if transstadially maintained pathogenic *F. t. holarctica* retain virulence during mosquito development. Mice were infected with homogenates of adult mosquitoes that had been exposed to *F. t. holarctica* during their aquatic larval stage. Treated mice developed tularemia within a few days. The results prove that transstadially maintained *F. t. holarctica* retain both viability and virulence during mosquito development. Moreover, the results indicate mosquitoes to have a more active role in the initiation and propagation dynamics of natural *F. t. holarctica* outbreaks than was previously thought[2].

Results

Mosquito infection rate and bacterial load. A total of 140 mosquitoes were exposed while in their larval stage to *F. t. holarctica* FSC200. Approximately one month later, when developed into flying adults, they were individually homogenized. In a PCR screen for *F. tularensis* specific *lpnA* gene, of 140 mosquito homogenates prepared, 33 tested positive resulting in an infection rate of approximately 24%. The 33 mosquito homogenates that tested positive for *F. tularensis* specific sequence in the preliminary PCR screen were pooled into seven groups for virulence studies in mice. Viable counts on selective agar plates did not reveal any growth of *F. t. holarctica* in any of the mosquito homogenates. Spiking experiments using *F. t. holarctica* in mosquito homogenate showed that real-time PCR identification of the *F. tularensis lpnA* gene, based on C_t values, was possible for concentrations of bacteria above 500 cfu per ml. The range of linearity was determined between 10^3 and 10^5 bacteria per ml.

Mosquito mediated transmission of *F. t. holarctica* to susceptible hosts. Mice were infected with PCR-positive homogenates from mosquitoes exposed, in their larval stage, to *F. t. holarctica*, and monitored for clinical signs of disease for 24 days. Seven pools of mosquito homogenates were used to infect eight mice (Table 1). Three of the eight mice developed clinical signs of disease within five days. The bacterial load of *F. t. holarctica* in mouse spleen from diseased mice ranged from 2.1×10^8 to 2.5×10^8 cfu per ml, Table 1. One of the homogenate pools was divided and injected to two different mice, both of which acquired tularemia with a bacterial load of $2.2 \pm 0.1 \times 10^8$ bacteria per ml in spleen. A potential correlation between real-time PCR C_t value and clinical signs of tularemia was not possible to investigate since the number of bacteria in the homogenates was below the range of linearity of the real-time PCR assay. Infection with homogenate from naïve (unexposed) mosquitoes did not result in any clinical signs of disease in mice. The positive control mice infected with *F. t. holarctica* FSC200 (17 cfu) exhibited clinical signs at day five when the bacterial load in spleen was determined to be $4.1 \pm 1.34 \times 10^8$ cfu per ml.

Discussion

Clinical experience and epidemiological data have strongly implied mosquito-borne transmission as the major transmission route for tularemia in Finland and Sweden[11–17]. Adult mosquitoes have been thought to transfer *F. t. holarctica* mechanically between susceptible hosts, so acting as amplifiers of ongoing outbreaks[2]. However, mosquito mediated outbreaks of human tularemia have also occurred during years when weak rodent populations contained no or few diseased animals[7,8] indicating that mechanical transmission may not be the only role of mosquitoes in tularemia transmission. Recent investigations, using molecular methods, have shown that mosquitoes exposed to *F. t. holarctica* during their aquatic larval stage maintain the bacterium or its DNA until the adult stage[3,4]. In this study, the injection of homogenates of mosquitoes that had been exposed to *F. t. holarctica* in their larval stage, resulted in tularemia in mice, thus demonstrating that the transstadially maintained *F. t. holarctica* is viable and virulent. Although vector borne transmission of bacterial pathogens is well documented in other types of vectors[19] this is, to our knowledge, the first evidence of a transstadially maintained bacterial pathogen that retains its virulence during the development of mosquitoes from larvae to adults.

During an ongoing outbreak, diseased and dead animals, with up to 10^{11} bacteria per ml of blood[4,20], contaminate the environment (water and soils) resulting in local hot spots of *F. t. holarctica*[21]. Field investigations, laboratory studies and genetic data indicate that the bacterium may persist in the environment for prolonged periods of time (several years)[7–10,22–26]. Our results suggest the possibility of biological transmission of the bacterial pathogen, and that the mosquito may play a role as the vector in outbreak initiation by reintroducing *F. t. holarctica* from environmental reservoirs into conditions favorable for growth, i.e. susceptible hosts.

Despite extensive attempts, we could not culture *F. t. holarctica* from the mosquito homogenates that were later proven to cause disease in mice. Mosquito antimicrobial peptides may inhibit the growth of *F. t. holarctica*. Insect antimicrobial peptides have previously been shown to inhibit the growth of *F. novicida* in a fly model involving *Drosophila melanogaster*[27]. Consequently, it was not possible to determine the bacterial load per mosquito through viable counts. However, the onset of disease in mice infected with *F. t. holarctica* is largely dependent on the infectious dose[20]. Here, the onset of disease appeared in the test mice and in the positive controls at a similar time after infection. Since the control mice had been administered an infectious dose of 17 cfu *F. t. holarctica* it is reasonable to assume that the number of bacteria in a mosquito was within the range of doses infectious to humans, which has been reported to be below 10 cfu[26].

Considering the high proportion of field-collected adult mosquitoes previously reported to test positive for *F. tularensis* (20%–30% of pooled samples)[4,28] there are comparatively few human cases of mosquito mediated human tularemia (average rate of 3.5 and 4.1 cases per 100 000 in Sweden and Finland, respectively, during the period 2007–2011)[18] indicating that only a small proportion of *F. t. holarctica*-positive mosquitoes transmit the disease. It has been suggested that transmission of tularemia may require inoculation by crushing of the infected mosquito on the skin, followed by rubbing or scratching. The limited transmission rate to man might be explained by putative differences in mosquito vector competence in terms of the

Table 1 | Infection experiment in which homogenates of mosquitoes exposed in the larval stage to fully virulent *F. t. holarctica*, unexposed mosquitoes, and a positive control (*F. t. holarctica* FSC200, 17 cfu) were used to infect mice. Mice injected *i.p.* were monitored for signs of disease for 24 days and the bacterial load of *F. t. holarctica* in spleens were determined. p.i.: post infection, n.a.: not applicable, n.d.: not detected

	Mosquito pools (pool size)	Mice	Diseased mice (days p.i.)	Bacterial load in spleen bacteria/ml
Mosquito batch 1	4 (4–5)	5	2 (5, 5)	2.3 and 2.1×10^8
Mosquito batch 2	3 (3)	3	1 (4)	2.5×10^8
Unexposed mosquitoes	4 (5)	4	*n.d.*	*n.d.*
F. t. holarctica	*n.a.*	3	3 (5, 5, 5)	3.2, 3.1 and 6.0×10^8

specific mosquito species' ability to acquire, maintain and transmit tularemia. It is well known from studies of other vector-borne diseases that the successful transmission of infectious agents depends on a complex interaction between the vector, the local environment, the agent and the susceptible hosts, and that one major factor in this complex interplay is the mosquito's vector competence for a specific pathogen that can vary considerably between different mosquito species[29]. An analysis of wild-caught mosquitoes from an area in Sweden where tularemia is endemic indicated the presence of *F. t. holarctica* in 11 different mosquito species[4]. *Ae. aegypti* or other mosquito species may be more or less competent vectors for the transmission of *F. t. holarctica* when taking a blood meal from a susceptible host. The relevance of *Ae. aegypti* as a model to study the transmission of *F. t. holarctica* in Sweden is open to question since the species does not occur naturally in Sweden. However, *Ae. aegypti* is one of the most established and tractable mosquito species for laboratory studies, and to our knowledge, there is no laboratory mosquito model available for any of the species native to Sweden. Thus, in order to determine the relevance of different mosquito species for tularemia transmission, the data presented here on *Ae. aegypti* need to be complemented with information regarding vector competence among locally occurring mosquito species.

The results of this study are in support of the hypothesis that a direct link exists between *F. t. holarctica* in aquatic habitats, via transstadial maintenance in mosquitoes, and transmission to susceptible mammals. The bacteria are associated with the mosquito in a passive, non-replicating quiescent state, and are resuscitated upon contact with the mammalian host, a process which represents a novel transmission cycle for a bacterial pathogen.

Methods

Mosquito breeding and exposure of mosquito larvae to *F. t. holarctica*. Eggs of the tropical mosquito *Ae. aegypti*, kindly provided by Oxitec (Oxitec LTD, Oxford, England), were hatched in deionized water. Larvae, approximately twenty per container (Mosquito Breeder, BioQuip Products, Rancho Dominguez, CA, USA), were maintained in tap-water at room temperature (RT) and fed crushed fish flakes. *F. t. holarctica* strain FSC 200[30] was grown on modified Thayer-Martin agar plates[31] at 37°C in 5% CO_2. Mosquito larvae were exposed to *F. t. holarctica* by transferring 2nd instar larvae into tap-water containing bacteria (FSC200) at a concentration of 10^7 colony forming units (cfu) per ml. After 24 h, the larvae were washed three times in tap-water and transferred to and kept in fresh tap-water until they emerged as flying adult mosquitoes when they were harvested by freezing at −70°C for 5 minutes and stored frozen. Mosquitoes exposed to *F. t. holarctica* in the larval stage were harvested on two occasions six days apart, viz. Day 1 (designated Batch 1 harvested 23 days after exposure) and Day 6 (designated Batch 2 harvested 30 days after exposure).

Mosquito homogenate. Mosquitoes exposed to *F. t. holarctica* in the larval stage were prepared as homogenates according to a method optimized for maintaining bacterial viability. A 1.5 ml Eppendorf tube containing a single mosquito, five 2 mm metal beads (Retsch, Haan, Germany) and 100 µl 0.9% NaCl, was shaken for 40 sec at 30 Hz using an MM400 mixer mill (Retsch, Haan, Germany). All homogenates were visually checked for complete homogenization of the mosquito.

Preliminary real-time PCR screen. The mosquito homogenates were screened for the presence of the *F. tularensis* specific *lpnA* gene using real-time PCR and the iQFt1 primer pair as previously described[32]. No DNA extraction was performed prior the PCR. Each reaction mixture comprised 1 µl mosquito homogenate, 10 µl SsoFast EvaGreen (BioRad Laboratories, Hercules, CA), 0.4 µl of each primer (20 pM), and MiliQ water to produce a total volume of 20 µl. An initial denaturation at 98°C for 2 min was followed by 45 cycles of 98°C for 5 s and 60°C for 5 s on an iCycler (Bio-Rad). To test the limit of detection and generate a standard curve for assessing target DNA concentrations, mosquito homogenates spiked with *F. t. holarctica* concentrations ranging from 5×10^1 to 5×10^5 cfu per ml were analyzed, in triplicates.

Mouse model. The mosquito homogenates that tested positive for *F. tularensis* DNA in the preliminary PCR screen were pooled before being used to infect mice to test for the viability and virulence of any *F. t. holarctica* present in the samples. Three to five homogenates were pooled to form seven pools ranging in volume from 250 µl to 300 µl. The seven pools were injected intra peritoneally (i.p.) into eight mice (200 µl/mouse), pool one being divided and injected into two different mice (120 µl each). Four pools of mosquito homogenates from unexposed mosquitoes were used as negative controls (200 µl/mouse). Three mice infected with 17 cfu (in a volume of 200 µl 0.9% NaCl) of *F. t. holarctica* strain FSC 200 were used as positive controls. The mice were monitored for clinical signs of disease for 24 days. The C57Bl/6 mice (in-house bred) were acclimatized for at least seven days under conventional conditions before infection. The study was approved by the Local Ethical Committee on Laboratory Animals in Umeå, Sweden, and all methods were performed in accordance with the ethical permission.

Mouse spleens were homogenized in 500 µl 0.9% NaCl and screened for presence of the *F. tularensis* specific *lpnA* gene using real-time PCR and the iQFt1 primer pair as previously described[32]. No DNA extraction was performed prior to the PCR. Each reaction mixture comprised 1 µl spleen homogenate template, 10 µl SsoFast EvaGreen (BioRad), 0.4 µl of each primer (20 pM), and MilliQ water to produce a total volume of 20 µl. An initial denaturation at 98°C for 2 min was followed by 45 cycles of 98°C for 5 s and 60°C for 5 s on an iCycler (Bio-Rad). All samples were analyzed in duplicate.

Culture methods. To determine the growth of *Francisella* in mosquito homogenates and in mice, serial dilutions of homogenized mosquitoes and mouse spleens were plated onto modified Thayer-Martin agar plates and incubated in 5% CO_2 for 3–10 days. Cultures were confirmed as *F. tularensis* using the PCR assay described above.

1. Olsufiev, N. in *Hum. Dis. with Nat. foci* (Pavlovsky, Y.) 219–281 (Foreign languages publishing house, 1966).
2. Petersen, J. M., Mead, P. S. & Schriefer, M. E. Francisella tularensis: an arthropod-borne pathogen. *Vet. Res.* **40**, 7 (2009).
3. Lundstrom, J. O. *et al.* Transstadial Transmission of Francisella tularensis holarctica in Mosquitoes, Sweden. *Emerg. Infect. Dis.* **17**, 794–799 (2011).
4. Thelaus, J. *et al.* Francisella tularensis Subspecies holarctica Occurs in Swedish Mosquitoes, Persists Through the Developmental Stages of Laboratory-Infected Mosquitoes and Is Transmissible During Blood Feeding. *Microb. Ecol.* **67**, 96–107 (2014).
5. Keim, P., Johansson, A., Wagner, D. M. & Epidemiology, M. Molecular epidemiology, evolution, and ecology of Francisella. *Ann. N. Y. Acad. Sci.* **1105**, 30–66 (2007).
6. Federal select agent program (APHIS & CDC). *Select Agents and Toxins List.* Date of acces: 20/11/2014, http://www.selectagents.gov/SelectAgentsandToxinsList.html.
7. Broman, T. *et al.* Molecular Detection of Persistent Francisella tularensis Subspecies holarctica in Natural Waters. *Int. J. Microbiol.* **2011**, 1–26 (2011).
8. Svensson, K. *et al.* Landscape epidemiology of tularemia outbreaks in Sweden. *Emerg. Infect. Dis.* **15**, 1937–1947 (2009).
9. Larsson, P. *et al.* Molecular evolutionary consequences of niche restriction in Francisella tularensis, a facultative intracellular pathogen. *PLoS Pathog.* **5**, e1000472 (2009).
10. Johansson, A. *et al.* A respiratory tularemia outbreak caused by diverse clones of Francisella tularensis. *Clin. Infect. Dis.* 1–22; DOI:10.1093/cid/ciu621(2014).
11. Olin, G. Occurence and mode of transmission of tularemia in Sweden. *Acta Pathol. Microbiol. Scand.* **19**, 220–247 (1942).
12. Christenson, B. An outbreak of tularemia in the northern part of central Sweden. *Scand. J. Infect. Dis.* **16**, 285–290 (1984).
13. Eliasson, H. *et al.* The 2000 tularemia outbreak: a case-control study of risk factors in disease-endemic and emergent areas. Sweden. *Emerg. Infect. Dis.* **8**, 956–60 (2002).
14. Eliasson, H. & Bäck, E. Tularaemia in an emergent area in Sweden: an analysis of 234 cases in five years. *Scand. J. Infect. Dis.* **39**, 880–9 (2007).
15. Eliasson, H., Broman, T., Forsman, M. & Bäck, E. Tularemia: current epidemiology and disease management. *Infect. Dis. Clin. North Am.* **20**, 289–311, ix (2006).
16. Rydén, P. *et al.* Outbreaks of tularemia in a boreal forest region depends on mosquito prevalence. *J. Infect. Dis.* **205**, 297–304 (2012).
17. Rossow, H. *et al.* Risk factors for pneumonic and ulceroglandular tularaemia in Finland: a population-based case-control study. *Epidemiol. Infect.* **142**, 2207–2216 (2014).
18. European Center of Disease Prevention and Control. *Annual epidemiological report 2013. Reporting on 2011 surveillance data and 2012 epidemic intelligence data.* (2013). Date of access: 20/11/2014. http://www.ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?List=4f55ad51-4aed-4d32-b960-af70113dbb90&ID=989.
19. Walker, D. *et al.* Emerging bacterial zoonotic and vector-borne diseases: Ecological and epidemiological factors. *JAMA* **275**, 463–469 (1996).
20. Molins, C. R. *et al.* Virulence Differences Among Francisella tularensis Subsp. tularensis Clades in Mice. *PLoS One* **5**, e10205 (2010).
21. Rossow, H. *et al.* Experimental Infection of Voles with Francisella tularensis Indicates Their Amplification Role in Tularemia Outbreaks. *PLoS One* **9**, e108864 (2014).
22. Parker, R. R., Steinhaus, E. A. & Kohls, G. M., J. W. Contamination of natural waters and mud with Pasteurella tularensis and tularemia in beavers and muskrats in the northwestern United States. *Bull. Natl. Inst. Health* **193**, 1–161 (1951).
23. Jellison, W. L. Tularemia in Montana. *Mont. Wildl.* 5–24 (1971).
24. Hopla, C. E. The ecology of tularemia. *Adv. Vet. Sci. Comp. Med.* **18**, 25–53 (1974).
25. Abd, H., Johansson, T., Golovliov, I., Sandström, G. & Forsman, M. Survival and growth of Francisella tularensis in Acanthamoeba castellanii. *Appl. Environ. Microbiol.* **69**, 600–6 (2003).

26. Dennis, D. T. *et al.* Tularemia as a biological weapon: medical and public health management. *JAMA* **285**, 2763–73 (2001).
27. Vonkavaara, M. *et al.* Francisella is sensitive to insect antimicrobial peptides. *J. Innate Immun.* **5**, 50–9 (2013).
28. Triebenbach, A. N. *et al.* Detection of Francisella tularensis in Alaskan Mosquitoes (Diptera: Culicidae) and Assessment of a Laboratory Model for Transmission. *J. Med. Entomol.* **47**, 639–648 (2010).
29. Desenclos, J.-C. Transmission parameters of vector-borne infections. *Médecine Mal. Infect.* **41**, 588–93 (2011).
30. Svensson, K. *et al.* Genome Sequence of Francisella tularensis subspecies holarctica Strain FSC200, Isolated from a Child with Tularemia. *J. Bacteriol.* **194**, 6965–6966 (2012).
31. Sandström, G., Tärnvik, A., Wolf-Watz, H. & Löfgren, S. Antigen from Francisella tularensis: nonidentity between determinants participating in cell-mediated and humoral reactions. *Infect. Immun.* **45**, 101–6 (1984).
32. Thelaus, J. *et al.* Influence of nutrient status and grazing pressure on the fate of *Francisella tularensis* in lake water. *FEMS Microbiol. Ecol.* **67**, 69–80 (2009).

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Author contributions

S.B. and J.N. are co-first authors having contributed equally to the work being described. S.B. performed all mosquito breeding, mosquito exposures, culture and real-time PCR analysis. J.N. coordinated and performed the infection experiment and contributed to writing the manuscript. S.B. and J.N. developed and performed the method for mosquito homogenates. M.F. contributed to writing and proofreading of the manuscript. J.T. coordinated and drafted the manuscript. All the authors participated in the experimental design and read and approved the final manuscript.

Additional information

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