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 aegypti 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.

V
ector-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.

Tularemia is a bacterial zoonotic disease of the northern hemisphere, endemic in certain geographical areas where it affects a wide range of mammals. 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. 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. 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. 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.

In Sweden and Finland, clinical experience and epidemiological data indicate that mosquitoes are the main transmission route of human tularemia. In fact, Finland and Sweden repeatedly report among the highest number of tularemia cases worldwide. 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. 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. 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. Attempts to culture the bacteria from such mosquitoes, and to experimentally transfer the infection to mice through mosquito bites, have so far been unsuccessful. 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.

**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 lpaN 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. 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 lpaN gene, based on C<sub>t</sub> values, was possible for concentrations of bacteria above 500 cfu per ml. The range of linearity was determined between 10<sup>2</sup> and 10<sup>9</sup> 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<sup>10</sup> to 2.5 × 10<sup>10</sup> 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 ± 0.1 × 10<sup>8</sup> bacteria per ml in spleen. A potential correlation between real-time PCR C<sub>t</sub> value and clinical signs of disease 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.

**Discussion**

Clinical experience and epidemiological data have strongly implied mosquito-borne transmission as the major transmission route for tularemia in Finland and Sweden. Adult mosquitoes have been thought to transfer F. t. holarctica medically between susceptible hosts, so acting as amplifiers of ongoing outbreaks. However, mosquito mediated outbreaks of human tularemia have also occurred during years when weak rodent populations contained no or few diseased animals 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. 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 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<sup>11</sup> bacteria per ml of blood, contaminate the environment (water and soils) resulting in local hot spots of F. t. holarctica. Field investigations, laboratory studies and genetic data indicate that the bacterium may persist in the environment for prolonged periods of time (several years)5-10,22-26. 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. 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. 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.

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)15 indicating that only a small proportion of F. 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<sup>8</sup> |
| Mosquito batch 2 | 3 (3) | 3 | 1 (4) | 2.5 × 10<sup>8</sup> |
| 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<sup>8</sup> |
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. 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. *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 was grown on modified Thayer-Martin agar plates at 37°C in 5% CO₂. 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 adults.

**Mosquito homogenate***

Mosquitoes, house bred) were acclimatized for at least seven days under conventional conditions (20°C, 50–60% relative humidity) and were infected with *F. t. holarctica* in the larval stage. The mosquito homogenates were screened for *F. tularensis* using the PCR assay described above. 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***

Four pools of mosquito homogenates from unexposed mosquitoes were used as controls in disease-endemic and emergent areas. Sweden. *Emerg. Infect. Dis.* 15, 1937–1947 (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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