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Francisella tularensis Subspecies *holarctica* Occurs in Swedish Mosquitoes, Persists Through the Developmental Stages of Laboratory-Infected Mosquitoes and Is Transmissible During Blood Feeding

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Abstract In Sweden, mosquitoes are considered the major vectors of the bacterium *Francisella tularensis* subsp. *holarctica*, which causes tularaemia. The aim of this study was to investigate whether mosquitoes acquire the bacterium as aquatic larvae and transmit the disease as adults. Mosquitoes sampled in a Swedish area where tularaemia is endemic (Örebro) were positive for the presence of *F. tularensis* deoxyribonucleic acid throughout the summer. Presence of the clinically relevant *F. tularensis* subsp. *holarctica* was confirmed in 11 out of the 14 mosquito species sampled. Experiments performed using laboratory-reared *Aedes aegypti* confirmed that *F. tularensis* subsp. *holarctica* was transstadially maintained from orally infected larvae to adult mosquitoes and that 25 % of the adults exposed as larvae were positive for the presence of *F. tularensis*-specific sequences for at least 2 weeks. In addition, we found that *F. tularensis* subsp. *holarctica* was transmitted to 58 % of the adult mosquitoes feeding on diseased mice. In a small-scale in vivo transmission experiment with *F. tularensis* subsp. *holarctica*-positive adult mosquitoes and susceptible mice, none of the animals developed tularaemia. However, we confirmed that there was transmission of the bacterium to blood vials by mosquitoes that had been exposed to the bacterium in the larval stage. Taken together, these results provide evidence that mosquitoes play a role in disease transmission in part of Sweden where tularaemia recurs.

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Introduction

Francisella tularensis, the etiological agent of the zoonotic disease tularaemia, is a Gram-negative coccobacillus. At present, *F. tularensis* is divided into four subspecies (*F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *mediasiatica*) [1, 2]. However, the taxonomic boundaries of *Francisella novicida* have recently been debated [3, 4]. Two subspecies are of clinical importance: type A (*F. tularensis* subsp. *tularensis*) and type B (*F. tularensis* subsp. *holarctica*) [2]. Subspecies *F. tularensis* strains are limited to North America, whilst *F. tularensis* subsp. *holarctica* strains exist throughout the Northern Hemisphere [2]. The clinical expression of tularaemia largely depends on the route of entry of the infectious agent [5].

The ecology of *F. tularensis* and how the bacterium persists between outbreaks is not clear; indeed, the historic focus on the epidemiology of tularaemia may have skewed our views [6]. Numerous wildlife species and several potential vectors (i.e. fleas, flies, lice, midges, mites, mosquitoes and ticks) have been associated with transmission of the bacteria [5, 7]. Outbreaks and occurrences of tularaemia are often patchy, i.e. it frequently occurs within natural foci in geographically

restricted areas, typically in association with just one or a few key mammalian and arthropod species. It has also been suggested that subspecies of *F. tularensis* differ with respect to their ecological niches [5]. Historically, the distribution of human tularaemia caused by *F. tularensis* subsp. *holarctica* has been associated with close proximity to water [8]. There is also evidence that *F. tularensis* subsp. *holarctica* strains persist in watercourses [9, 10], possibly in association with protozoa [11–14]. Even though mosquitoes are considered to be mechanical vectors of *F. tularensis* [7], it is generally thought that they are the major route of transmission for human cases of tularaemia in Sweden [15, 16], and the occurrence of infected mosquitoes (*Aedes cinereus*) was reported as early as 1942 [17]. Recent detection of *F. tularensis* subsp. *holarctica* deoxyribonucleic acid (DNA) in adult mosquitoes, reared from larvae collected in an endemic area, implies a novel transmission cycle originating in the aquatic habitats of mosquito larva [18]. However, the dynamics of outbreaks of tularaemia remain unknown.

The aim of this study was to evaluate further the role of mosquitoes as vectors of *F. tularensis* subsp. *holarctica* by investigating the occurrence of the bacterium in mosquito populations in a Swedish area with endemic tularaemia. Further, experimental exposure and transmission were investigated using a fully virulent *F. tularensis* subsp. *holarctica* strain in a laboratory colony of the tropical mosquito *Aedes aegypti*.

Methods

Occurrence of *F. tularensis* subsp. *holarctica* in Field-Sampled Mosquitoes

Study Area, Mosquito Sampling and Species Identification In 2003 tularaemia re-emerged in Örebro County (59° 16′ 0″ N, 15° 12′ 0″ E, a population of 281,100, 2011). Before 2000, only a handful of cases had been reported from the county; however, between 2003 and 2005, 229 human cases of tularaemia were reported (http://www.smi.se/in-english/statistics/tularaemia/). Mosquito sampling points were chosen based on the information supplied by local physicians about the geographical distribution of human tularaemia cases.

Adult mosquitoes were sampled using the Centre for Disease Control (CDC) miniature light traps with carbon dioxide as an additional attractant, as previously described [19]. From June to September in 2004, mosquitoes were sampled on eight sampling occasions, each comprising three successive nights. Sampling was performed at four locations (Ormesta, Karlslund, Vattenparken and Kårsta) within the Örebro Region, and three traps were used at each location. The CDC traps were placed in trees approximately 1.5 m above the ground and were activated early in the evening and run for 12–14 h. Trapped mosquitoes were anaesthetized with carbon dioxide, separated into three random groups, placed in plastic ampoules (each containing 1 to 500 mosquitoes) and euthanized by freezing on dry ice. The three different groups were used for the following: (1) an initial PCR screening for *F. tularensis*, (2) mosquito species identification and (3) attempts to cultivate the bacteria. Ampoules containing mosquitoes were stored at −70 °C until further analysis.

During species identification, the mosquitoes were kept cold on a chill table, illuminated by a cold light lamp and identified to species based on their morphology [20]. Mosquitoes identified to species were sorted into ampoules according to species, capture date and trap and were returned to the −70 °C freezer.

Molecular and Culture-Based Methods for F. tularensis Subsp. holarctica Detection in Field-Sampled Mosquitoes Prior to DNA extraction from mosquitoes collected in the field, 250 μl distilled water and 12.5 μl 2.8 M NH_4OH were added to the ampoules of a maximum of 50 mosquitoes. The mixture was incubated at room temperature (RT) for 10 min. The samples were vortexed for 20 s and, thereafter, bead beaten to disrupt the mosquito bodies and release intracellular bacteria (450 mg each of 1 and 0.1 mm silica beads for 60 s, MiniBeadBeater-8, BioSpec). Bead-beaten samples were incubated at 95 °C for 30 min before centrifugation at 800×*g* for 3 min. DNA was extracted using phenol (pH 8)–chloroform–isoamyl alcohol (25:24:1) and subsequently precipitated using ethanol. The pellet was resuspended in 40 μl of distilled water and frozen if not immediately subjected to PCR analysis.

The screening of ampoules of mosquitoes for the presence of *F. tularensis* was performed using a real-time PCR probe-based assay for detection of the *F. tularensis*-specific *lpnA* gene, using iQFt1 F/R primers and probe as previously described [13] (Table 1). Positive samples were subjected to a *F. tularensis* subsp. *holarctica*-specific PCR based on the 30-bp deletion region and fragment size analysis according to the literature [18, 21]. Each reaction consisted of 1 μl template, 1× AmpliTaq Gold PCR buffer, 40 μM each of the primers FtM19InDel (Table 1), 2.6 mM $MgCl_2$, 1 M betaine, 0.2 mM dNTP, 0.5 U of AmpliTaq Gold Polymerase and Milli-Q water to a total volume of 25 μl. An initial denaturation at 94 °C for 2 min was followed by 50 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C 30 s, followed by final incubation at 72 °C for 5 min on MyCycler (Bio-Rad Laboratories, Hercules, CA, USA). Positive control reactions using DNA from *F. tularensis* and negative control reactions without a template were included in each PCR run. Genomic rat control DNA and internal control primers IQFPrat36B4 and IQRPrat36B4 were used as previously described [13] (Table 1). The resulting amplicons were sized by capillary electrophoresis on the CEQ™ 8800 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA, USA). A volume of 1 μl PCR products was mixed with CEQ DNA Size Standard Kit 400 in a sample loading solution according to the manufacturer's manual; a fragment size of 100 bp corresponds to *F. tularensis* subsp. *holarctica*.

Table 1 Primers used in this study

Primer	Target	Sequence	Ref
iQFt1 F iQFt1 R	*lpnA*	5′-CGCAGGTTTAGCGAGCTGTT-3′ 5′-GCAGCTTGCTCAGTAGTAGCTGTCT-3′	[13]
iQFt1 p		FAM 5′-CATCATCAGAGCCACCTAACCCTA-3′	
lpnA2F lpnA2R	*lpnA*	5′-CGCAGGTTTAGCGAGCTGTT-3′ 5′-GAGCAGCAGCAGTATCTTTAGC-3′	[18]
FtM19InDel F FtM19InDel R	30-bp deletion	Wellred-PA 5′ CCAGTACAAACTCAATTTGGTTATCATC-3′ 5′-GTTTCAGAATTCATTTTTGTCCGTAA-3′	[9]
iQFPrat36B4 iQRPrat36B4	Internal control	5′-GCCCAGAGGTGCTGGACAT-3′ 5′-ATTGCGGACACCCTCTAGGA-3′	[13]
iQrat36B4 p		TET 5′-ACAGAGCAGGCCCTGCACACTCG-3′	
ITS1_F338 ITS1_R427	Mosquito	CGCTCGGACGCTCGTAC CTTCGAGCTTCGACGACACA	[28]

A subset of the third group of the mosquitoes collected in the field was homogenized and spread on selective and non-selective cysteine heart agar plates supplemented with sheep blood, CHAB-PACCV and CHAB [22]. Selective and non-selective agar medium according to the literature [23] was also used in parallel. The selective supplements used were 600,000 IU of penicillin, 1,000,000 IU of polymyxin B and 100 mg of cycloheximide. All agar plates were incubated at 37 °C in 5 % CO_2. Culture plates were checked for potential *Francisella* colonies after 2 days of incubation. Potential *Francisella* colonies were picked and analyzed with PCR using the MicroSeq 500 16S rRNA Bacterial Identification Kit (Applied Biosystems).

The infection rate (IR) and confidence intervals of *F. tularensis* in field-sampled mosquitoes were calculated using bias-corrected likelihood methods and skew-corrected score [24].

A Laboratory Mosquito Model for Studies of Transstadial Maintenance and Transmission of Virulent *F. tularensis* Subsp. *holarctica*

Francisella Strains and Constructs *F. tularensis* subsp. *holarctica* FSC 200 [25] was grown on modified Thayer–Martin agar plates [26] at 37 °C in 5 % CO_2. The amino acid sequence of *Discosoma* sp. red fluorescent protein, DsRed-Express (Clontech Laboratories, Inc., Mountain View, CA, USA) was converted into a DNA sequence with optimized codon usage for protein expression in *F. tularensis* and chemically synthesized by GenScript, NJ, USA. The 684-bp sequence was introduced into the *Nde* I-*Eco* RI site of pKK289Km-*gfp* [11] under control of the LVS GroESL promoter, after removal of the *gfp* gene. The resulting plasmid pKK289Km-DsRed was then introduced to *F. tularensis* subsp. *holarctica* FSC200 by cryotransformation [27]. The resulting plasmid-carrying strain was denoted FSC 849. All experiments using FSC 200 and FSC 849 were performed under BSL 3 conditions.

Rearing of Mosquito A. aegypti Eggs of the tropical mosquito *A. aegypti*, kindly provided by Oxitec (Oxitec Ltd., Oxford, England), were hatched in deionized water. Larvae, approximately 20 per container (Mosquito Breeder, BioQuip Products, Rancho Dominguez, CA, USA), were maintained in tap water at 26 °C and fed crushed fish flakes. When they developed, pupae were removed from the larval containers and placed in clean tap water. Adult mosquitoes were fed from cotton wool soaked in 10 % dextrose solution.

Exposure of Mosquito Larvae to F. tularensis Subsp. holarctica and Transstadial Maintenance Uptake of *F. tularensis* subsp. *holarctica* by the mosquito larvae and transstadial maintenance to the resulting pupae and adult mosquitoes was studied using DsRed-labelled *F. tularensis* subsp. *holarctica* (FSC 849) (Fig. 1). Mosquito larvae (second instar) were transferred to tap water containing bacteria at a concentration of 10^7 colony-forming units (cfu) per ml. After a 24-h period of exposure to bacteria, the larvae were washed three times in water and transferred to fresh tap water where they were kept until harvested (by freezing −70 °C for 5 min). Harvesting was performed at one of the three developmental stages: larva (fourth instar), pupa or adult mosquito. In addition, haemolymph from pupae and adults were obtained through perfusing the thorax using capillary tubes. All samples (larva, pupa, adult and haemolymph) were stored at −70 °C until further analysis for the presence of *F. tularensis* using real-time PCR.

Mosquitoes and Artificial Blood Meal Experiments Adult *A. aegypti* mosquitoes, exposed to *F. tularensis* subsp. *holarctica* as larvae (as described above), were presented with an artificial blood meal (Fig. 1). A small vial containing sheep, guinea pig or mouse blood was covered with parafilm and placed upside down on the mosquito container, representing an artificial source of blood. Transmission of bacteria from the mosquito to the blood was investigated using *F. tularensis*-specific real-time PCR with lpnA2 primers (Table 1), culturing and immunofluorescence microscopy.

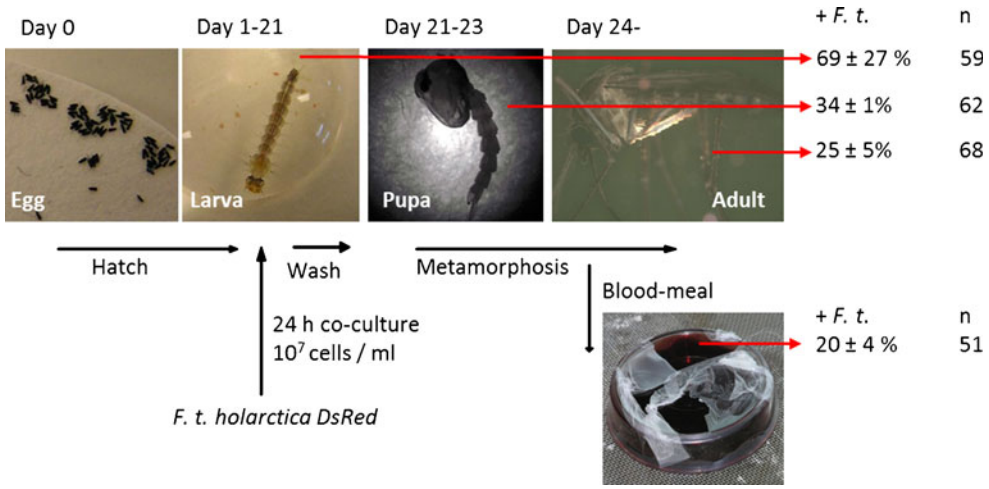

Fig. 1 Experimental set-up for investigating the uptake and transstadial maintenance of *F. tularensis* subsp. *holarctica* in the mosquito *A. aegypti*. Percent of *F. tularensis*-positive mosquitoes during different developmental stages (fourth instar larva, pupa and adult mosquito). Data are summarized from three separate experiments involving between 59 and 68 mosquitoes in each run. Transmission of the transstadially maintained *F. tularensis* subsp. *holarctica* was investigated after mosquito feeding from an artificial source of blood. The mosquitoes that were exposed to *F. tularensis* subsp. *holarctica* as larvae were allowed to feed from blood vials. The results are presented as percent of blood vials positive for *F. tularensis* DNA after mosquito feeding

In another experiment, nine batches (one to eight individuals) of uninfected adult mosquitoes were allowed to feed on vials of mouse blood spiked with approximately 10^6 cfu per ml (FSC 849). Mosquitoes were harvested within 48 h after feeding. In both experiments, blood-fed mosquitoes were harvested by freezing, as described above, and analyzed for the presence of *F. tularensis* using real-time PCR.

Mosquitoes and Transmission Experiments Using Rodent Host In order to study potential uptake and transmission of *F. tularensis* subsp. *holarctica* by mosquitoes to susceptible hosts, C57Bl/6 mice were used (in-house bred). Mice were housed under conventional conditions and allowed to acclimatize for at least 7 days before infection. The study was approved by the Local Ethical Committee on Laboratory Animals in Umeå, Sweden. With the aim of determining the optimal time for mosquitoes to feed on blood of infected mice, bacterial counts in mouse blood during *F. tularensis* subsp. *holarctica* infection were investigated. For these studies, five mice were subcutaneously (s.c.) infected with *F. tularensis* subsp. *holarctica* (FSC 200, infection dose 27 cfu). Bacterial counts in mouse blood were monitored daily. Blood from infected mice was bead beaten, serially diluted and spread on Thayer–Martin agar plates [26] incubated at 37 °C in 5 % CO_2. Bacterial growth was recorded by counting cfu. All transmission experiments with infected mosquitoes and mice were performed under BSL 3 conditions.

Prior to exposure to the mosquitoes in the transmission study, mice were anesthetized by intra-peritoneal injection of ketamine (Ketalar® vet) (75 mg/kg) and medetomidine (Domitor® vet) (1 mg/kg) and placed on top of a mosquito container to allow the mosquitoes to feed on mouse blood. All mice were removed from the mosquito container when either ten mosquitoes had fed (as observed by extended blood-filled abdomen) or 30 min had passed. The experiment evaluated the possibility that *A. aegypti* could transstadially transmit *F. tularensis* subsp. *holarctica*. Here, 184 female mosquitoes exposed to *F. tularensis* subsp. *holarctica* at the larval stage (as described above) were allowed to feed on 14 naïve mice (Fig. 2(a)). As negative controls, non-infected mosquitoes fed on five naïve mice. Mice exposed to mosquitoes were monitored for signs of disease for 25 days.

Another experiment was designed to evaluate the ability of *A. aegypti* to transmit *F. tularensis* subsp. *holarctica* between susceptible hosts. Here, 33 non-exposed female *A. aegypti* divided into five batches were allowed to feed on two mice (Fig. 2(b)). The mice had been infected with *F. tularensis* subsp. *holarctica* s.c. (FSC 849, infection dose 27) 3 days prior to the mosquito blood meal. Four days after the mosquitoes fed on the infected mice, they were allowed to feed on five naïve mice (Fig. 2(b)). Mice were observed for symptoms of the disease for 21 days. Mosquitoes from the two experiments were harvested after the blood meal and analyzed for the presence of the bacteria using *F. tularensis*-specific real-time PCR and lpnA2 primers (Table 1).

Detection of *F. tularensis* Subsp. *holarctica* in the Laboratory Mosquito Model

DNA Extraction Tris–EDTA (TE) buffer (20–100 μl) was added to mosquito larvae, pupae and haemolymph samples

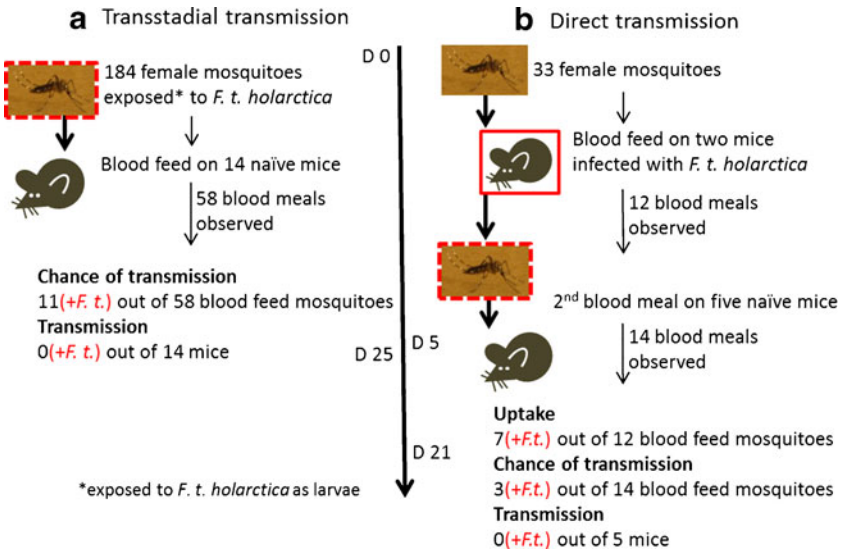

Fig. 2 Experiment to investigate the ability of the mosquito *A. aegypti* to transstadially and directly transmit *F. tularensis* subsp. *holarctica* to susceptible hosts. *a* Transstadial transmission of *F. tularensis* subsp. *holarctica*: mosquitoes exposed to the bacterium as larvae were allowed to feed on the blood of naïve mice. Chance of transmission is the number of mosquitoes that took a blood meal and were later identified as positive for *F. tularensis*. Transmission is the number of mice positive for *F. tularensis* at the end of the experiment. *b* Mosquito-mediated direct transmission of *F. tularensis* subsp. *holarctica* between hosts: mosquitoes were allowed to feed on the blood of mice infected with *F. tularensis* subsp. *holarctica*. Five days later, the mosquitoes were allowed to feed on blood of naïve mice in order to establish whether there was direct transmission. Uptake is the number of mosquitoes that took blood from infected mice and were later identified as positive for *F. tularensis*. In both experiments, mice were monitored for signs of disease for up to 25 days, and no traces of *F. tularensis* were detected in mouse spleen or blood samples analyzed using real-time PCR and culture methods (transmission). Mosquitoes were analyzed for the presence of *F. tularensis* and mouse DNA using PCR (uptake and chance of transmission)

and boiled for 15 min. DNA from adult mosquitoes was extracted using the SoilMaster DNA Extraction Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The resulting DNA pellet was resuspended in 50 μl TE buffer. Blood samples (50 μl) were prepared using the Qiagen Tissue Kit, adding 150 μl G2 and 2 μl proteinase K, and were incubated at 65 °C for 15 min and then extracted using the EZ1 Advanced PrepRobot (Qiagen, Venlo, Netherlands) and EZ1 Tissue Kit (Qiagen). The elution volume was 50 μl.

PCR Analysis Samples from the mosquito experiments were screened for the presence of *F. tularensis* using a modified real-time PCR SYBR-based assay for detection of the *F. tularensis*-specific *lpnA* gene, as previously described [18]. Each reaction volume consisted of 1–5 μl template, 10 μl 1× SsoFast EVA Green (Bio-Rad), 400 nmol/l each of the lpnA2R/F primer (Table 1) and Milli-Q up to 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 and a melting curve of 60–95 °C on CFX96 (Bio-Rad). In addition, an internal control was included using a construct of a non-coding part of the pSKluxCDABE (ATG: Biosynthetics) in plasmid pKK214tet, flanked by the lpnA2 primer pair sequence generating a 93-bp fragment (CGCAGGTTTAGCGAGCTGTTTAGATTTCG AGTTGCAGCGAGGCG GCAAGTGAACGAATCCCCA GGAGCATAGCTAAAGATACTGCTGCTGCTC).

In order to confirm the material from *A. aegypti* in samples, specific primers ITS1-F338 and ITS1-R427 (Table 1) were used as previously described [28].

To test the detection limit for the real-time PCR assay and to generate a standard curve for assessing target DNA concentrations in the matrixes analyzed, DNA extraction was performed in triplicate on samples of larvae, pupae, haemolymph and adults spiked with *F. tularensis* subsp. *holarctica* concentrations ranging from 1×10^0 to 1×10^6 cells per ml.

Culture of F. tularensis Subsp. holarctica Cultures from laboratory-reared *A. aegypti* mosquitoes and from vials of blood used for the artificial blood meals were produced from samples that had been homogenized in screw-capped microtubes with integral o-ring seals using 1-mm silica beads and the Mini-BeadBeater-8 (BioSpec Products, Inc., Bartlesville, OK, USA). The resulting samples were spread on selective T4 agar plates [29] and incubated at 37 °C in 5 % CO_2. Bacterial growth was recorded after 3 to 12 days of incubation depending on the degree of contamination. Potential *Francisella* colonies were picked and confirmed using the PCR SYBR-based assay for detection of the *F. tularensis*-specific *lpnA* gene described above.

Fluorescence Microscopy To confirm transmission of *F. tularensis* to the vials of blood used to feed artificially the mosquitoes exposed to *F. tularensis* subsp. *holarctica* in the

larval stage, fluorescence microscopy was performed using the Leica DMR (UV light, 330–380 nm). Blood was smeared on glass slides and allowed to dry before analysis either by direct detection of fluorescence from *DsRed* (using FSC849) or by incubation with a primary rabbit anti-*F. tularensis* antibody (Grünow, R.) (1:200) for 2 h at RT. The glass slides were thereafter rinsed five times in dH_2O before incubation with a secondary FITC-labelled mouse anti-rabbit antibody (1:1,000) for 1 h at RT. The glass slides were then rinsed five times with dH_2O before microscopy.

Results

Mosquito Abundance and Occurrence of *F. tularensis* in Wild-Caught Mosquitoes

The first group of mosquitoes collected in the field was used for the initial PCR screening for the presence of *F. tularensis*. This material comprised approximately 13,500 mosquitoes in 188 mixed species ampoules (Table 2). The number of mosquitoes caught at the four locations on each sampling occasion peaked twice (Fig. 3). The peak in June (week 23) corresponds to the emergence of snow-pool mosquito species (mosquito functional group 2a), while the peak in August (week 33) corresponds to the emergence of flood water species (mosquito functional group 2b) [19].

F. tularensis-specific sequences (*lpnA*) were detected in mosquito ampoules collected throughout the summer season and at all four sampling locations (Table 2). The *F. tularensis* IR in the mixed species mosquito pools increased approximately 4–6 weeks after the peak occurrence of snow-pool mosquitoes and, again, approximately 2–4 weeks after the peak occurrence of flood water mosquitoes (Fig. 3). This demonstrates that the infection rate of *F. tularensis* in the mosquito population varies over the summer, and it increases towards the end of summer with the declining mosquito population comprising an increasing proportion of flood water mosquitoes.

F. tularensis Subsp. *holarctica* in Several Species of Wild-Caught Mosquitoes

The second group of mosquitoes collected in the field was used for species identification and comprised originally of approximately 9,000 individuals in 181 ampoules. The material was identified to species and sorted by sampling occasion, location and species, resulting in 859 ampoules (data not shown). In order to investigate the presence of *F. tularensis* subsp. *holarctica* in wild-caught mosquitoes and any potential association between the bacterium and a specific mosquito species, a subset of the species sorted sample was analyzed further. The subset was selected in order to maximize the chance of finding *F. tularensis*-positive mosquitoes. The selection was performed on the basis of the initial PCR screening of the first group of mosquitoes collected in the field, during which high frequencies of *F. tularensis* positives were detected in the mixed species mosquito ampoules from the Ormesta area on four sampling occasions (i.e. weeks 27, 29, 35 and 37, see Table 2). This subset of species identified mosquitoes comprised of 791 mosquitoes in 89 single-species ampoules (Table 3). Twenty of the 89 mosquito ampoules were positive for *F. tularensis*-specific sequences (*lpnA*, Table 3). Eighteen of the 20 *lpnA*-positive ampoules were also positive in the screening for *F. tularensis* subsp. *holarctica* using the FtM19InDel primer pair that targets the *F. tularensis* subsp. *holarctica*-specific 30-bp deletion region (resulting in a 100-bp amplicon) (Table 3). Of the 14 mosquito species identified in the subset, 11 were found to be positive for *F. tularensis* subsp. *holarctica* sequences. These 11 mosquito species belonged to the four mosquito genera: *Aedes*, *Anopheles*, *Coquillettidia* and *Culex* (Table 3).

Table 2 The first group of the mosquitoes collected from Örebro, an area of Sweden with endemic tularaemia, was analyzed for the presence of the *F. tularensis*-specific sequence *lpnA*. Samples were collected from four locations (Ormesta, Karlslund, Vattenparken and Kårsta) on eight sampling occasions (June to September)

Sampling occasion	Total ampoules (individuals)	*F. tularensis*-positive ampoules (individuals)
W 23	41 (5,666)	2 (66)
Ormesta	19 (3,810)	n.d.
W 25	27 (1,882)	n.d.
Ormesta	8 (960)	n.d.
W 27	21 (968)	2 (70)
Ormesta	8 (549)	1 (60)
W 29	19 (740)	3 (125)
Ormesta	6 (376)	1 (70)
W 31	22 (1,316)	2 (47)
Ormesta	7 (635)	n.d.
W 33	25 (1,839)	1 (70)
Ormesta	6 (465)	n.d.
W 35	19 (778)	4 (143)
Ormesta	5 (98)	3 (63)
W 37	14 (287)	2 (85)
Ormesta	5 (126)	2 (85)
Total	188 (13,476)	16

The numbers refer to the number of ampoules containing mosquitoes that were analyzed, and those within brackets refer to the estimate of the total number of individuals within the ampoules. Results of the analysis of material from all four locations are presented for each sampling week (W 23 to 37). Results of analysis of the material from Ormesta are presented separately for each sampling week (Ormesta) since they were the basis for selecting dates and locations for further analysis

n.d. not detected

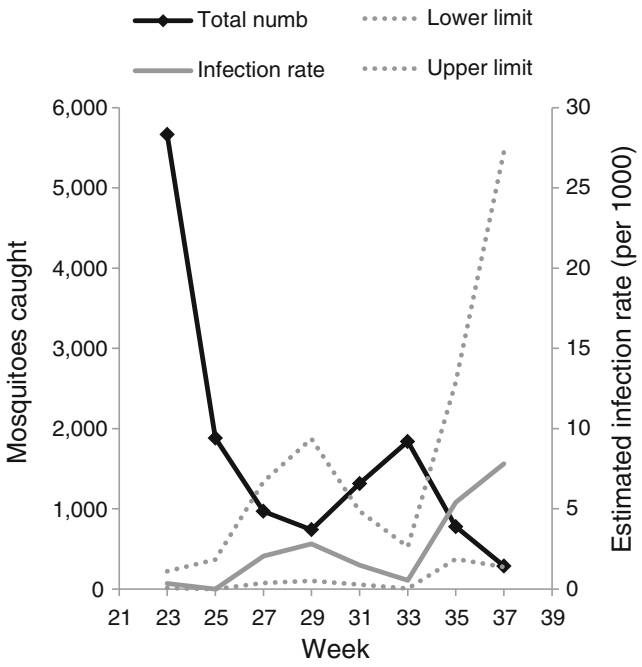

Fig. 3 The first group of the mosquitoes collected in the field that was subjected to an initial PCR screening for the presence of *F. tularensis*. The number of mosquitoes caught on each sampling occasion in relation to the estimated IR per 1,000 mosquitoes collected. *Dotted lines* represent the calculated upper and lower IR limits. The *F. tularensis* infection rate were highest at 2 to 6 weeks after the peak in total number of mosquitoes

Attempts to culture *Francisella* performed on the third group of mosquitoes collected in the field did not result in any identification of *Francisella* colonies.

Transstadial Maintenance of *F. tularensis* Subsp. *holarctica* in Laboratory-Reared *A. aegypti*

A. aegypti mosquitoes exposed to *F. tularensis* subsp. *holarctica* as larvae were harvested at different developmental stages (larvae, pupae and adults) and analyzed for the presence of *F. tularensis* using molecular methods (i.e. the *lpnA* PCR assay, Fig. 1). Spiking experiments showed that real-time PCR detection of the *F. tularensis lpnA* gene (lpnA2 primers) was possible for concentrations of >200 cells per ml in all matrixes (*F. tularensis* subsp. *holarctica* in mosquito larvae, pupae and adults). The range of linearity for all matrixes was determined to be between 10^3 and 10^6 *F. tularensis* subsp. *holarctica* cfu/ml.

Real-time PCR analysis of mosquito larvae exposed to *F. tularensis* subsp. *holarctica* showed that 5 days after infection, 69±27 % of the larvae (total of 59 analyzed) were positive for *F. tularensis* (Fig. 1). Approximately 1 week later, the remaining exposed larvae developed into pupae. In the PCR analysis of a subset of the pupae, 34±1 % of the 62 pupae examined were positive for the presence of the bacterium. From

Table 3 The second group of the field-sampled mosquito material was species identified. A subset of this material was subjected for further analysis for the presence of *F. tularensis* subsp. *holarctica*. The subsample consisted of ampoules of mosquitoes captured in the Ormesta area on four sampling occasions W 27, 29, 35 and 37 (chosen based on results presented in Table 2, as it was found that samples from Ormesta collected during these weeks had the highest frequency of *F. tularensis*-positive ampoules)

Mosquito species	Total ampoules (individuals)	*F. tularensis* positive	*F. tularensis* subsp. *holarctica* positive
A. cinereus	18 (410)	4 (146)	4
Aedes sticticus	10 (33)	3 (11)	3+
Aedes vexans	5 (5)	1 (1)	1
Anopheles claviger	6 (8)	2 (3)	1
Anopheles maculipennis	7 (16)	2 (5)	1
Coquillettidia richiardii	10 (153)	3 (45)	3
Culiseta morsitans	4 (7)	n.d.	–
Culex pipiens/*Culex torrentium*	6 (28)	1 (1)	1
Aedes annulipes	5 (11)	1 (1)	1
Aedes cantans	9 (86)	1 (13)	1
Aedes communis	4 (11)	n.d.	–
Aedes intrudens	3 (19)	1 (1)	1
Aedes leucomelas	1 (2)	1	1+
Aedes punctor	1 (2)	n.d.	–
Total	89 (791)	20	18

The numbers refer to the number of ampoules containing mosquitoes that were analyzed, and those within brackets refer to the estimate of the total number of individuals within the ampoules. The presence of *F. tularensis*-specific sequences was determined, and positive samples were further investigated for the presence of the clinically relevant subspecies *F. tularensis* subsp. *holarctica* using fragment size analysis of a 30-bp deletion region unique to *F. tularensis* subsp. *holarctica* (FtM19Indel); + indicates the presence of *F. tularensis* subsp. *holarctica* and other *F. tularensis* subspecies

n.d. not detected

the remaining pupae, adult mosquitoes emerged ($n=68$). Approximately 14–16 days after the original exposure of mosquito larva, 25±5 % of adult mosquitoes were positive for *F. tularensis*. These results are based on three independent experiments, each starting with 59 to 68 mosquito larvae.

In addition, haemolymph from pupae and adult mosquitoes was sampled and analyzed in order to confirm the presence of *F. tularensis* inside the mosquitoes. Approximately 29±4 % of the haemolymph from pupae ($n=62$) and 19±2 % of the haemolymph from adult mosquitoes ($n=68$) were positive for *F. tularensis*.

In order to investigate the possibility that mosquitoes take up and subsequently maintain viable *F. tularensis* subsp. *holarctica* derived from a blood meal, nine batches of previously uninfected mosquitoes (one to eight mosquitoes in each batch) were allowed to feed from vials containing mouse blood spiked with *F. tularensis* subsp. *holarctica* (approximately 10^6 cfu per ml). Mosquitoes from five out of the nine batches were, when newly fed (analyzed within 48 h of the blood meal), found positive for *F. tularensis* using real-time PCR. However, attempts to culture the bacterium from mosquitoes were unsuccessful.

Transstadial Transmission of *F. tularensis* Subsp. *holarctica* to Blood After Artificial Mosquito Feeding

Batches of approximately 20 mosquitoes exposed to *F. tularensis* subsp. *holarctica* in the larval stage were allowed to feed from vials of blood from three different sources (sheep, guinea pig and mouse). A total of 1–11 mosquitoes were observed to feed from each vial of blood. Mosquito feeding was confirmed by PCR on blood from the vials using ITS1 primers targeting a mosquito specific sequence. Real-time PCR analysis of blood indicated the presence of *F. tularensis* in one out of six sheep blood vials, in five out of 25 guinea pig blood vials and in five out of 20 mouse blood vials. Overall, *F. tularensis* subsp. *holarctica* was transmitted to 20±4 % of the blood vials as a result of feeding by mosquitoes exposed to *F. tularensis* subsp. *holarctica* as larvae (Fig. 1). Transmission results were confirmed by fluorescence microscopy, which directly detected *F. tularensis* subsp. *holarctica* (*DsRed*) in the post-feeding blood vials (data not shown). The numbers of bacteria recorded were low, and efforts to cultivate the bacteria from mosquitoes and blood samples were unsuccessful.

Mosquito Transmission of *F. tularensis* Subsp. *holarctica* to Susceptible Hosts

Transstadial Transmission A total of 184 female *A. aegypti*, exposed to *F. tularensis* subsp. *holarctica* during the larval stage, were divided into 14 batches and allowed to feed on naïve mice ($n=14$) (Fig. 2(a)). In total, we observed 58 mosquito blood meals, 11 of which were by mosquitoes that were later shown to be positive for *F. tularensis* based on the presence of both mouse and *F. tularensis* DNA in the mosquito. The mice were monitored for a minimum of 15 days and a maximum of 25 days for clinical signs of disease. None of the mice developed tularaemia, and no traces of *F. tularensis* were detected in spleen or blood samples collected from the mice after they had been euthanized and analyzed using real-time PCR and culturing.

We speculated a potential requirement for prior activation of the bacteria, resulting from a blood meal, for mosquito transmission of *F. tularensis* subsp. *holarctica*. Thus, five of the mosquito batches (a total of 37 mosquitoes exposed to *F. tularensis* subsp. *holarctica* in the larval stage) were allowed a first blood meal on naïve mice ($n=5$) 5 days prior to a second blood meal. Thirteen mosquitoes were fed during this first run. None of the mice developed tularaemia, and no bacteria were detected in blood or spleen samples from these mice.

The negative controls, four mice bitten by non-infected mosquitoes, displayed no signs of illness during the entire experiment (21 days). The positive controls, five mice infected (s.c.) with *F. tularensis* subsp. *holarctica*, were euthanized on day 3 or 4 after infection due to severe symptoms.

Direct Transmission The possibility of mosquitoes as vectors for *F. tularensis* subsp. *holarctica* transmission between diseased and naïve hosts was investigated. Counts of *F. tularensis* subsp. *holarctica* in the blood during mouse infection were observed to increase from $5\pm4\times10^2$ cfu/ml blood 2 days after infection to $4\pm4\times10^{10}$ bacteria/ml blood 4.5 days after infection when the experiment was terminated due to clinical signs of the disease (Fig. 4). In order to

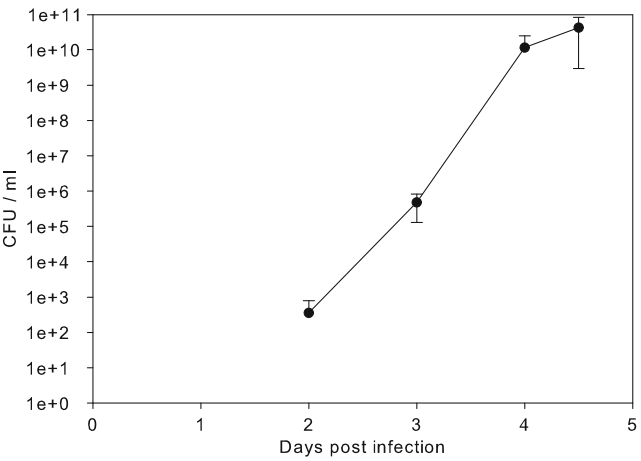

Fig. 4 Bacterial counts of *F. tularensis* subsp. *holarctica* in blood during mouse infection. Colony-forming units (*cfu*) of the bacterium *F. tularensis* subsp. *holarctica* in the mouse blood following subcutaneous injection (infectious dose of 27 cfu/ml)

investigate the possibility of direct transmission, the mosquitoes were allowed to feed on mice on the 3rd day of *F. tularensis* subsp. *holarctica* infection. Thirty-three uninfected female mosquitoes divided into five batches were allowed to blood feed on mice ($n=2$) infected with *F. tularensis* subsp. *holarctica* (27 cfu of FSC 849, Fig. 2(b)). At the time of mosquito feeding, bacterial counts in the blood of infected mice were determined to be $4\pm0.5\times10^5$ cfu/ml, similar to what is observed during mouse infection with wild-type *F. tularensis* subsp. *holarctica* (Fig. 4). Out of 12 observed blood meals, seven lead to transmission of the bacteria to the feeding mosquito (infection rate of 58 %). Mosquitoes that acquired *F. tularensis* subsp. *holarctica* through blood feeding maintained the bacteria for at least 2 weeks. However, transmission of the disease was not observed upon a second blood meal on five naïve mice, where 14 out of the 33 mosquitoes were observed to take a blood meal. Four of the mosquitoes that took a second blood meal and fed on the naïve mice were later found to be positive for *F. tularensis*.

Discussion

F. tularensis subsp. *holarctica* circulates in the mosquito population in Örebro, a Swedish area with endemic tularaemia. Sequences specific for *F. tularensis* (*lpnA*) were detected in mosquitoes from all sampling locations in the area and throughout the summer season (June to September). Almost all *F. tularensis* (*lpnA*)-positive samples subjected for further analysis were also positive for the *F. tularensis* subsp. *holarctica*-specific region (FtM19InDel). Thus, sequences specific to the clinically relevant *F. tularensis* subsp. *holarctica* were found in mosquitoes of several different species. Considering recent reports of a high diversity of *Francisella* in environmental samples [9, 30–32], these results indicate a restriction of *Francisella* diversity through persistence in mosquitoes. In line with this, *F. novicida* has been shown not to be transstadially maintained during mosquito development [33]. This suggests selectivity associated with the pathogenic *F. tularensis* subsp. *holarctica* persistence in mosquitoes.

Traditionally, mosquitoes have mainly been considered to function as mechanical vectors for transmission of *F. tularensis* subsp. *holarctica* between hosts [7]. We have previously shown that the bacterium can be transstadially maintained, from larvae to adult, in mosquitoes of several species collected as larvae in the field and reared to adults in the laboratory [18]. Here, a model for mosquito exposure, where *A. aegypti* larvae were feed on planktonic *F. tularensis* subsp. *holarctica*, was established in order to investigate the role of a potential mosquito vector in the life cycle of the bacterium. Our results show that *F. tularensis* subsp. *holarctica* is transferred to mosquito larvae from its aquatic habitat and transstadially maintained in 25 % of the adult mosquitoes. Further, the bacterium is transmissible to the blood when mosquitoes feed on artificial sources of blood (i.e. blood in vials). However, none of the mammals exposed to the *F. tularensis*-positive mosquitoes were infected during the transmission experiment. It is very likely, however, that transmission of tularaemia by mosquitoes in nature occurs at much lower frequencies than could be detected in this study. Here, out of the 72 mosquitoes that fed on the blood of naïve mice, a total of 14 were later confirmed to be positive for *F. tularensis*. We speculate that the frequency of *F. tularensis* subsp. *holarctica* transmission by mosquitoes in the environment is low and probably depends on species-specific interaction alternative that transmission is mechanical and requires inoculation via smashing of vector onto the skin. There was no association between *F. tularensis lpnA* and any specific mosquito species in the material collected in the field. Mosquito species positive for *F. tularensis* included both snow-pool species (four positive out of 23 ampoules, infection rate of 0.17) and flood water species (eight positives out of 33 ampoules, infection rate of 0.24). The initial screening of the first group of mosquitoes collected in the field revealed an increasing IR of *F. tularensis* with declining mosquito populations in the late August and September. This coincides largely with human cases of tularaemia, which were reported in the area from August to October 2004 (a total of 50 cases, http://www.smi.se/in-english/statistics/tularaemia/?y=2004#statistics-nav). The relevance of using *A. aegypti* as a model to study the transmission of *F. tularensis* subsp. *holarctica* in Sweden is open to question since the species does not occur naturally in Sweden. Nevertheless, to our knowledge, there is no suitable laboratory mosquito model available for any of the species native to Sweden. *Aedes* sp. or other mosquito species may be more or less competent vectors for transmission of *F. tularensis* subsp. *holarctica* to susceptible hosts. In order to confirm that a transmission route for *F. tularensis* subsp. *holarctica* from the environment (water, soil or host) via a mosquito vector is relevant for transmission of the disease, vector competence of relevant locally occurring mosquito species needs to be demonstrated.

The frequency of *F. tularensis*-positive mosquitoes after feeding from infected mice was higher than the frequency of infection of mosquitoes exposed to *F. tularensis* subsp. *holarctica* as larvae (approximately 58 and 25 %, respectively). This indicates the possibility of mosquito-mediated transmission of *F. tularensis* subsp. *holarctica* directly between susceptible hosts during an ongoing outbreak. It is possible that the mosquitoes play a dual role as vectors during outbreaks of tularaemia, both as a link between a reservoir and susceptible host and as an amplifier of an ongoing outbreak by direct transmission of the bacterium between susceptible hosts.

Sampling of haemolymph from pupae and adult mosquitoes exposed to *F. tularensis* subsp. *holarctica* as larvae showed a high frequency of *F. tularensis* subsp. *holarctica*-positive

samples (29 and 19 %, respectively), suggesting that mosquitoes used in the model do maintain *F. tularensis* subsp. *holarctica*, internally. Based on our real-time PCR assay, we did not, however, find any evidence that the bacterium replicates in the mosquito *A. aegypti*. The real-time PCR assay used in this study can detect low numbers (in hundreds) of *F. tularensis* in mosquitoes, but the range of linearity was established between 10^3 and 10^6 bacteria per mosquito. However, it cannot be excluded that the PCR assay detected DNA from non-viable bacteria.

The bacterial counts of *F. tularensis* subsp. *holarctica* reached approximately 10^{10} bacteria/ml in mouse blood during infection. Consequently, a deceased animal can contaminate the local environment (soil and water) with an extremely high concentration of *F. tularensis* subsp. *holarctica*. Even though the retention time for the bacterium varies depending upon a variety of factors such as temperature, salinity, direct exposure to sunlight and other physical factors that generally affect the survival of microbes, it has been documented that surface waters and mud in stream bottoms and ponds can be contaminated with *F. tularensis*. The bacterium has been shown to survive at infective levels in those substrates for many months [10]. Other studies have shown that although *F. tularensis* subsp. *holarctica* persists locally in the environment for prolonged periods (in years), under specific conditions, the bacterium is reported to enter a non-infective, viable but non-culturable (VBNC) state [9, 13, 34, 35]. Hot spots of recently introduced infective bacteria may act as temporary sources for tularaemia outbreaks via dispersal of the bacterium as aerosols (contaminated dust) or contaminated water. However, the role of such hotspots in the perpetuation of *F. tularensis* subsp. *holarctica* is unclear. Previously published data suggest that *F. tularensis* subsp. *holarctica* persists in association with water and protozoa and thus could be transported up through the microbial food web to mosquito larvae [11]. Moreover, insect antimicrobial peptides have been shown partially to inhibit the growth of *F. novicida* in a fly model involving *Drosophila melanogaster* [36]. The increasing body of whole-genome data from *Francisella* species and subspecies indicate that there is almost no exchange of genetic material between *F. tularensis* subspecies [37]. This could be due to them occupying separate environmental niches or having different life cycles and suggests that the bacteria replicate in environments where there is little opportunity to interact with other bacteria. In addition, an epidemiological investigation combined with high-resolution genotyping of *F. tularensis* subsp. *holarctica* isolates obtained from patients in the Örebro region indicated that genetic subpopulations of the bacteria were present throughout the summer season and also persisted over several years with little genetic variation [38]. Taken together, current data indicate that between outbreaks, the causative agent of tularaemia, *F. tularensis* subsp. *holarctica*, persists in the environment in a dormant state with little or no replication.

In conclusion, we have shown that *F. tularensis* subspecies *holarctica* is found in several genera and species of wild-caught mosquitoes in Sweden. Subsequent laboratory studies of *A. aegypti* larvae exposed to fully virulent *F. tularensis* subsp. *holarctica* demonstrated transstadial maintenance of the bacteria during metamorphosis into adult mosquitoes. The possibility of mosquito transmission of the bacteria to blood vials during artificial feeding was confirmed as well as transmission of the bacteria from infected mice to mosquitoes. Under our experimental conditions, we did not demonstrate transmission from mosquitoes to mice that confirm the viability of the bacteria. We found no evidence for the replication of *F. tularensis* subsp. *holarctica* in mosquitoes, but the results from the molecular analysis indicate that the bacterium does reside inside mosquitoes.

To further clarify the transmission mechanism, future studies should investigate the potential of mechanical transmission of the bacterium via analysis of mouthparts after feeding on infected host and possible transmission via interrupted feeding. A very prolonged incubation of the susceptible hosts (i.e. longer than the 21 days used in this study) may also ensure that potential VBNC *Francisella* cells resuscitate. Further, *F. tularensis* infection models show a great variation in ID50 depending on the site of infection, an examination of the dermal site where vectors probe might be suitable to indicate local infection.

The ability of *F. tularensis* subsp. *holarctica* to be transstadially maintained through the mosquito life cycle, as demonstrated in this study, may have implications for the mode of transmission of the microorganism. We suggest that mosquitoes take up the bacteria during their larval stage and, once they become adults, transmit the disease, at very low frequencies, to a susceptible host where a massive burst of replication occurs. These susceptible hosts act as local amplifiers of *F. tularensis* subsp. *holarctica* and facilitate the spread of disease via vectors (including mosquitoes) and, after death, through carcasses contaminating the local environment. This plausible scenario involves the dynamics of at least two complex cycles: aquatic microbial dynamics that, via mosquito larvae, may lead to infection of mammals and terrestrial dynamics with potential arthropod vectors and mammal population densities that may favour the spread of the disease. Both cycles would exhibit common source–sink dynamics, with outbreaks during a limited time (i.e. months) resulting in massive growth of *F. tularensis* subsp. *holarctica* in susceptible hosts and thus passive contamination of the environment with *F. tularensis* subsp. *holarctica*, followed by bacterial long-term persistence and a slow decline over time. The need for the intertwining of these two complex cycles in space and time, as well as the specific biotic and abiotic factors governing the spread of disease, may explain the inherent unpredictability of tularaemia outbreaks and illustrates the need for further studies to elucidate the enigmatic life cycle of *F. tularensis*.

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