

Transmission of Severe Fever with Thrombocytopenia Syndrome Virus by *Haemaphysalis longicornis* Ticks, China

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We demonstrate maintenance and transmission of severe fever with thrombocytopenia syndrome virus by *Haemaphysalis longicornis* ticks in the larva, nymph, and adult stages with dissemination in salivary gland, midgut, and ovarian tissues. The *H. longicornis* tick is a competent vector to transmit this virus in both transovarial and transstadial modes.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by SFTS virus (SFTSV), identified in China in 2009 (1) and subsequently in South Korea (2) and Japan (3). Symptoms of SFTS usually include fever, thrombocytopenia, and leukocytopenia; case-fatality rates are 10%–30% (1,4). SFTS is implicated as largely a tick-associated disease, supported by evidence that many patients had exposure to ticks before disease onset (1). The longhorned tick, *Haemaphysalis longicornis*, the most abundant human-biting tick species in most SFTS-endemic areas of China (5), was found to harbor SFTSV (1,6,7). These studies suggested that *H. longicornis* ticks might be competent vectors for SFTSV transmission. Our study was designed to determine the role of the *H. longicornis* tick as a vector in maintenance and transmission of SFTSV.

The Study

We randomly allocated 90 female *H. longicornis* ticks from an SFTSV-free colony into 2 equal groups, experimental and control. We injected the experimental group with SFTSV and the control group with phosphate-buffered

saline (PBS). Seven days postinjection, we used 18 of the 35 live SFTSV-infected ticks for the detection of viral RNA by real-time reverse transcription PCR (rRT-PCR) (online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/5/15-1435-Techapp1.pdf>); all showed positive results, confirmed by subsequent rRT-PCR and sequencing analysis. Twelve days postinjection, we dissected 5 live ticks from the experimental group to detect SFTSV in salivary glands and ovaries by indirect fluorescence assay (IFA), which showed notable SFTSV-specific fluorescence (Figure 1, panel A). For the control group, none of the 19 ticks tested by rRT-PCR had SFTSV RNA, and none of the 5 ticks tested by IFA showed SFTSV-specific fluorescence (Figure 1, panel B).

We then let the remaining 12 live ticks from both groups feed on naive Balb/C mice (4 ticks/mouse) until the ticks detached from the mice. The engorged females were harvested and maintained to lay eggs. We determined transovarial transmission of SFTSV by further testing of SFTSV RNA from eggs, larvae, and nymphs using rRT-PCR. A total of 15 pools of eggs laid by 5 infected *H. longicornis* ticks (3 pools from each tick, each pool coming from a single female) were SFTSV RNA positive. In contrast, the egg pools from ticks of the control group were all negative. When hatched to larvae, 20 of 25 pools derived from the infected *H. longicornis* ticks (5 pools from each tick) tested positive for SFTSV RNA; all 25 larvae pools of the control group tested negative (Table 1).

We further performed transstadial transmission of SFTSV by rearing larvae to adults. All remaining larvae were reared to nymphs and adults by feeding on 20 naive Balb/C mice. We subjected 1 mouse to SFTSV RNA testing after it was bitten by each pool of larvae and the hatched nymphs and adults; we used the other mice for feeding multiple pools of larvae and the hatched nymphs and adults. We fed 3,195 larvae in the experimental group and 2,987 in the control group to engorgement and randomly selected and tested engorged larvae. We maintained the other engorged larvae for molting to nymphs. In all, 694 engorged larvae in the experimental group and 652 engorged larvae in the control group successfully molted to nymphs

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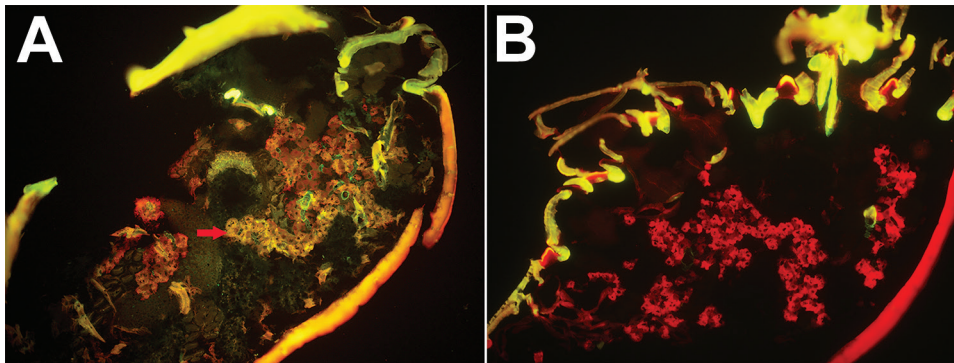


Figure 1. Specific detection of severe fever with thrombocytopenia syndrome virus (SFTSV) in microinjected *Haemaphysalis longicornis* ticks by indirect fluorescence assay. A) SFTSV-injected ticks 12 days after microinjection (original magnification $\times 10$). Red arrows indicate specific fluorescence s. B) Phosphate-buffered saline-injected ticks 12 days after microinjection (original magnification $\times 10$). No specific fluorescence is seen.

(online Technical Appendix Tables 1, 2). The remaining 569 nymphs in the SFTSV group and 527 nymphs in the control group were fed on 20 naive Balb/C mice; 453 nymphs in the SFTSV group and 437 in the control group were fully engorged. We divided the remaining engorged nymphs into 5 replicate cohorts; 166 engorged nymphs ($39\% \pm 6\%$ standard error [SE]) in the experimental group and 155 ($38\% \pm 5\%$ SE) in the control group matured to adults (online Technical Appendix Tables 1, 2). The overall hatching rate of eggs and molting rates of nymphs and adults in the 2 groups were comparable.

All 25 engorged larvae pools (5 pools from each mother) in the SFTSV-infected group and none from the control group were positive for SFTSV RNA (Table 1). After the larvae molted to nymphs, 23 of 25 nymph pools from the SFTSV-infected group and none from the control group tested positive for SFTSV RNA. Similarly, all 25 engorged nymph pools from the SFTSV-infected group and none from the control group were positive. When the second generation emerged, we tested 50 adults (25 females, 25 males) in each group for SFTSV RNA; in the SFTSV-infected group, 44% (11/25) of the females and 36% (9/25) of the males tested positive, whereas all 25 females and 25

males in the control group were negative. Positive samples were confirmed by identical sequences to that of the inoculated virus strain.

A total of 83 naive Balb/C mice were infested by ticks (online Technical Appendix Table 3). All 3 Balb/C mice fed by the SFTSV-infected females were positive for exposure to SFTSV 1 week after the ticks detached. Of the naive Balb/C mice that were bitten by larvae from the SFTSV-infected group, 4 of 5 were positive for SFTSV RNA, as were 4 of 5 mice bitten by nymphs, 4 of 5 mice bitten by adult female ticks, and 3 of 5 mice bitten by male ticks; mice bitten by ticks from the control group were negative (Table 2). We used IFA to test serum samples from the mice collected before and 3 weeks after detachment of ticks at different developing stages; all mice positive for SFTSV RNA demonstrated seroconversion against SFTSV (Table 2).

Three of 4 pools of saliva and hemolymph from the experimental group were SFTSV RNA positive. We selected 5 females at random from each group to detect SFTSV in tissues by IFA. The salivary glands, midguts, and ovaries of the SFTSV-injected group displayed SFTSV-specific fluorescence (Figure 2).

Table 1. Detection of severe fever with thrombocytopenia syndrome virus RNA in experimental and control *Haemaphysalis longicornis* ticks

Source	Experimental infection group		Control group	
	No. tested	% Positive \pm SE	No. tested	% Positive
Mother tick carcasses	5	100	5	0
Egg pool*	15	100	15	0
Larvae pool†	25	80.0 \pm 1.7	25	0
Engorged larvae pool‡	25	100	25	0
Nymph pools§	25	92.0 \pm 1.4	25	0
Engorged nymph	25	100	25	0
Male adult	25	36.0 \pm 5.4	25	0
Female adult	25	44.0 \pm 2.9	25	0
Female hemolymph pool¶	3	66.7 \pm 9.4	4	0
Female saliva pool#	4	75.0 \pm 14.4	4	0
Male hemolymph pool¶	3	33.3 \pm 9.4	4	0

*Eggs were tested in pools of 60.

†Larvae were tested in pools of 50.

‡Engorged larvae were tested in pools of 5.

§Nymphs were tested in pools of 5.

¶Hemolymph collected from 5 ticks was pooled as 1 sample.

#Saliva collected from 5 ticks was pooled as 1 sample.

Table 2. Detection of severe fever with thrombocytopenia syndrome virus in *Haemaphysalis longicornis* tick-infested mice*

Stage (sex)	No. mice	No. ticks/mouse	No. positive by rRT-PCR		No. positive by IFA	Titer \pm SE
			Experimental group	Control group		
Adults (female)	3	4	3	0	3	3.01 \pm 0.30
Larvae	5	50	4	0	4	2.78 \pm 0.15
Nymphs	5	10	4	0	4	3.16 \pm 0.17
Adults (female)	5	5	4	0	4	3.09 \pm 0.15
Adults (male)	5	5	3	0	3	2.81 \pm 0.35

*IFA, indirect fluorescence assay; rRT-PCR, real-time reverse transcription PCR.

We observed a significantly higher level of viral load in second-generation eggs than in second-generation adults ($p < 0.001$ by Mann-Whitney U-test). We also found a significantly higher level ($p < 0.0001$) of viral load in saliva of engorged second-generation adults than in saliva of unengorged adults, indicating that SFTSV had multiplied.

Conclusions

We report the experimental maintenance and transmission of SFTSV in *H. longicornis* ticks. After microinjection of SFTSV, the virus disseminated in ovaries and salivary glands. Infected *H. longicornis* ticks could transmit SFTSV successfully in both transovarial and transstadial modes. The appearance of SFTSV in saliva and hemolymph suggests that the virus circulates in the tick hemocoel and is expressed in saliva. In addition, naive Balb/C mice infested with experimentally infected adults, larvae, and nymphs all became infected, evidenced by both detection of SFTSV-specific RNA and seroconversion.

These findings, together with data on natural infection in the field (1,6), implicate *H. longicornis* ticks as competent vectors for SFTSV. However, the evidence derived from IFA and rRT-PCR tests could not indicate that the virus is infectious. More efforts should be taken to demonstrate the infectivity of SFTSV in the transmission cycle.

H. longicornis ticks are widely distributed in the Asia-Pacific region (8–12). Predominant hosts of *H. longicornis* ticks include humans, poultry, livestock, wild rodents, and birds (12–14). As displayed in mice in the current research, SFTSV is likely to be maintained through vertical and horizontal transmission in ticks that infest these wild and domestic mammals. This maintenance has been evidenced by an extraordinarily high prevalence of SFTSV in sheep, cattle, dogs, pigs, and other animals (7,14). In areas where *H. longicornis* ticks are endemic, infested animals could be considered as key reservoirs in maintaining and transmitting SFTSV (15). The close contact between animals and their owners could pose another way of acquiring infection, in addition to tick bites.

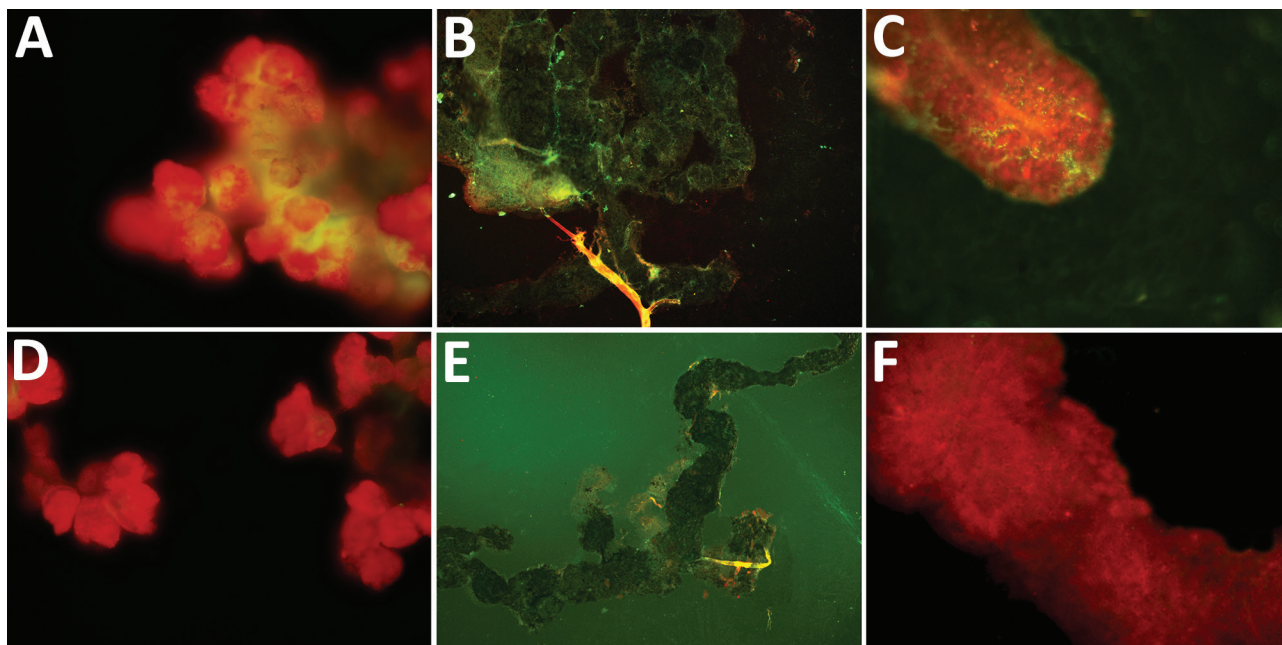


Figure 2. Specific detection of severe fever with thrombocytopenia syndrome virus (SFTSV) in tissues of adult *Haemaphysalis longicornis* ticks by indirect fluorescence assay. The green fluorescence represents the SFTSV virus. A) Salivary gland of SFTSV-injected tick (original magnification $\times 40$). B) Midgut of SFTSV-injected *H. longicornis* tick (original magnification $\times 10$). C) Ovary of SFTSV-injected tick (original magnification $\times 40$). D) Salivary gland of phosphate-buffered saline (PBS)-injected tick (original magnification $\times 40$). E) Midgut of PBS-injected *H. longicornis* tick (original magnification $\times 10$). F) Ovary of PBS-injected tick (original magnification $\times 40$).

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Transmission of Severe Fever with Thrombocytopenia Syndrome Virus by *Haemaphysalis longicornis* Ticks

Materials and Methods

SFTSV Strain and Culturing

The severe fever with thrombocytopenia syndrome virus (SFTSV) (Phlebovirus WCH/97/HN/China/2011, GenBank accession nos. JQ341190, JQ341189, and JQ341188, for L, M, and S segments) used in this study was isolated from a patient in Henan Province of China in 2011 (1), and maintained in the Vero E6 cell line with complete Dulbecco's modified Eagle's medium (DMEM), 10% fetal serum, and 10 U/mL penicillin and streptomycin. After we determined viral loads by quantitative real-time PCR, we harvested the virus for artificial infection of ticks by microinjection.

Tick Colony and Rearing

H. longicornis ticks were collected by flagging on vegetation in Shangcheng County, Henan Province, China in 2011. We established SFTSV-free tick colonies in our laboratory from engorged females. Briefly, the *H. longicornis* ticks were allowed to feed on Balb/C mice. All mice in this study were 2-week-old males, specific-pathogen free, supplied by the Center of Experimental Animals, Academy of Military Medical Sciences, China. The fully engorged females were kept individually until they laid eggs. We randomly sampled 10 batches (30 eggs in each batch) of eggs to screen for SFTSV, along with the corresponding adult mother tick, by isolation and RT-PCR assays, described later. The eggs from the groups in which both the mother tick and the filial eggs were negative for SFTSV were incubated to larvae. The larvae and

the following nymphs were fed on Balb/C mice, and the molted adults were subjected to the trial. The transmission cycle of SFTSV in *H. longicornis* ticks was simulated following the procedures shown in Technical Appendix Figure 1, with each step described as follows.

Artificial Infection of Ticks with SFTSV by Microinjection

Adult female *H. longicornis* ticks from the aforementioned SFTSV-free colony were infected with SFTSV by the microinjection protocol developed by Kocan et al. (2) with modification. We injected 1 μ L of virus culture (5.9×10^5 copies/mL) into each tick through its anal pore with a microsyringe (1 inch, 33 gauge needle) under a dissecting stereomicroscope (Technical Appendix Figure 2). We injected the same volume of phosphate-buffered saline (PBS) into ticks that were used as the control group. The ticks that were crawling and active after injection were maintained in an Intelligent Climate Cabinet (Saife Company, Ningbo City, China) with a relative humidity of $95 \pm 5\%$ at 22°C .

Transmission Cycle of SFTSV in Ticks

Two weeks after injection, the female ticks were fed on Balb/C mice so we could investigate transovarial transmission. The engorged female ticks were maintained until they laid eggs, which were allowed to hatch to larvae under the same conditions as described earlier. We screened subsequent larvae for SFTSV infection to assess the efficiency of transovarial transmission. Larvae and subsequent nymphs were allowed to feed on Balb/C mice until fully engorged and molt to nymphs and adults. At each developmental stage, ticks were starved for 3 weeks between molting and the next feeding. We tested the derived nymphs and adults for SFTSV to evaluate the efficiency of transstadial transmission.

Detection of SFTSV in Ticks of Different Developmental Stages

We subjected ticks of different developmental stages to real-time PCR and RT-PCR to determine their SFTSV infection status. We extracted RNA from egg pools (60/pool), larva pools (50/pool), nymph pools (5/pool) (Technical Appendix Table 2), and individual adult ticks

using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. For real-time PCR assay, we used the one-step Primer Script RT-PCR Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions with 1 μ L PCR primer mix (20 μ M of sense and antisense each), 0.5 μ L probe (10 μ M) and 2 μ L total RNA in LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). The real-time PCR primers (5'-ACCTCTTTGACCCTGAGTTWGACA-3' and 5'-CTGAAGGA GACAGGTGGAGATGA-3') and probe (5'-Hex-TGCCTTGACGATCTT-MGB-3) were targeted at the S-segment of the SFTSV (3). We performed RT-PCR and sequencing of the S-segment on positive samples (BNYS1-F: 5'-TCTTCTCCATCAAGAACAGC-3', BNYS1-R: 5'-TTCGACAAAATTAGACCTCC-3') to verify the real-time PCR results.

We prepared the positive control standard (nt. 1456–1557 of the SFTS virus segment S sequence, reference sequence GenBank accession no. KC505134) as described previously (4). We prepared serial dilutions from 10^8 to 10^3 copies/mL in diethylpyrocarbonate-treated water and stored them in RNase-free tubes at -80°C .

We performed quantification of SFTSV as described earlier in the 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Absolute RNA quantification was included in every assay and was generated by using RNA transcripts produced earlier. Standard curves included 5 dilutions and 3 replicate wells for each dilution. All samples were quantified in 3 replicate wells. Levels of SFTSV RNA concentrations were expressed as copies/mL.

Detection of SFTSV in Saliva and Hemolymph of Adult Ticks

To prepare the molted adult ticks to salivate, we allowed them to engorge partially by feeding on Balb/C mice. Saliva was collected (Technical Appendix Figure 3, panel A) from the engorged ticks as described previously (5). After saliva collection, we obtained hemolymph samples from each tick by clipping a front leg and placing the tip of a glass micropipette to the wound (Technical Appendix Figure 3, panel B). The saliva from 5 ticks in each group was

pooled and the hemolymph from the same 5 ticks was pooled, mixed with 140 μ L PBS, and subjected to the RNA extraction procedure using the QIAamp Viral RNA Mini Kit (QIAGEN). We then applied the RNA to RT-PCR assay, for which positive amplicons were sequenced as mentioned earlier.

IFA Detection of SFTSV in Ticks

Twelve days following injection with SFTSV, we embedded the whole bodies of the ticks in paraffin and cut them longitudinally at a cryostat (Leica CM 3050; Leica Microsystems, Wetzlar, Germany). We put the frozen slices on glass slides and subjected them to immunofluorescent assay (IFA) for SFTSV detection.

We then selected 10 females at random from the SFTSV and control groups and individually dissected their guts, salivary glands, and ovaries under sterile conditions using a dissecting microscope. After marginal cuts and scutum removal with lancets, we carefully removed the hemolymph around the tissues with filter papers and replaced it with sterilized PBS 3 times. We placed the tick tissues on glass slides and subjected them to IFA for SFTSV detection.

We soaked sheet glasses with prepared tissues in PBS with 5% skim milk to deparaffinize. We incubated the slices at 37°C for 1 hour with mAb that was previously prepared (6) in PBS with 0.05% Tween20. We used serum from Balb/C mice as a negative control. Following triple washing with PBS and 1 final washing with distilled water, we incubated the slices at 37°C for 30 minutes with fluorescence-conjugated goat antimouse antibodies (Zhongshanjinqiao, Beijing, China) at 1:100 dilution with Evans blue. We washed the slices in PBS 3 times and finally washed them with distilled water, visualizing with an Olympus BX51 Microscope until dried.

Detection of SFTSV in Mice

We collected serum samples from the mice 3 times (before tick feed, 1 week after tick engorgement, and 3 weeks after tick repletion) and extracted RNA using a QIAamp Viral RNA mini kit and detection by real-time PCR, as described earlier. By using the viral antigen of the SFTSV patient source from the Vero E6 cell line, we detected specific IgG against SFTSV by indirect IFA, as previously described. We measured antibody titers with serum dilution starting at 1:16 and then serially 2-fold to determine endpoint titers.

Statistical Analysis

The antibody reciprocal titers were log-transformed. We used the Mann-Whitney test to determine the difference of SFTSV viral load in generation 2 eggs and adults, as well as the difference between the viral load of unengorged generation 2 adults and saliva collected from engorged generation 2 adults.

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Technical Appendix Table 1. Numbers (\pm standard error) of generation 2 ticks used in each stage of the transmission cycle

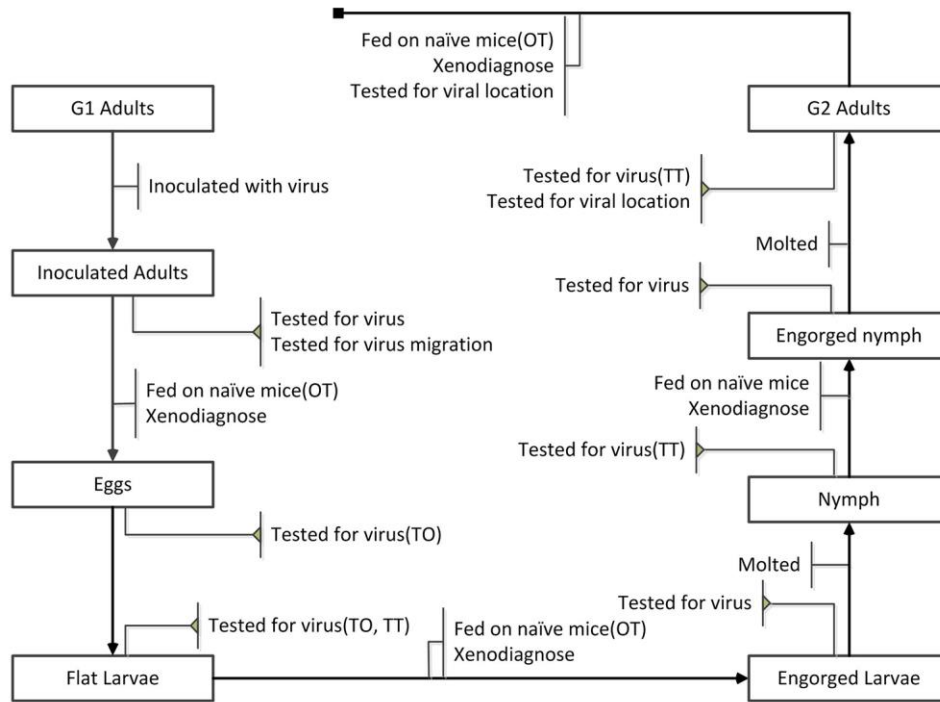
Source	SFTSV group	Control group
Eggs	1305.6 \pm 47.7	1436.6 \pm 159.9
Eggs for detection (eggs \times pools)	60 \times 3	60 \times 3
Eggs left	1125.6 \pm 47.7	1256.6 \pm 159.9
Hatched larvae	889 \pm 66.2	847.4 \pm 114.2
Larvae for detection (larvae \times pools)	50 \times 5	50 \times 5
Larvae left	639 \pm 66.2	597.4 \pm 114.2
Engorged larvae	185.2 \pm 24.5	179.4 \pm 5.6
Engorged larvae for detection (larvae \times pools)	5 \times 5	5 \times 5
Engorged larvae left	160.2 \pm 24.5	154.4 \pm 5.6
Nymphs	138.8 \pm 19.7	130.4 \pm 10.6
Nymphs for detection (nymphs \times pools)	5 \times 5	5 \times 5
Nymphs left	113.8 \pm 19.7	105.4 \pm 10.6
Engorged nymphs	90.6 \pm 14.9	87.4 \pm 10.7
Engorged nymphs for detection (nymphs \times pools)	5 \times 1	5 \times 1
Engorged nymphs left	85.6 \pm 14.9	82.4 \pm 10.7
Adults	33.2 \pm 6.1	31 \pm 4.6
Female adults	24.2 \pm 8.9	23.2 \pm 5.3
Female adults for detection	5	5
Hemolymph (females \times pools)	5 \times 1	5 \times 1
Saliva (females \times pools)	5 \times 1	5 \times 1
Male adults	9 \pm 4	7.8 \pm 2.5
Male adults for detection	5 \times 1	5 \times 1
Hemolymph (males \times pools)	5 \times 1	5 \times 1

Technical Appendix Table 2. Mean days in each period of development of *H. longicornis* ticks in the SFTSV study (\pm standard error)

Period	Days	
	SFTSV group	Control group
Preoviposition period	8.20 \pm 2.39	7.8 \pm 1.80
Oviposition period	7.73 \pm 1.69	7.7 \pm 1.72
Egg hatching period	38.50 \pm 1.24	38.4 \pm 1.46
Larva feeding period	3.52 \pm 0.64	3.4 \pm 0.63
Larva premolt period	14.50 \pm 1.85	14.2 \pm 2.37
Larva molting period	7.70 \pm 1.67	7.9 \pm 1.64
Nymph feeding period	5.80 \pm 1.28	5.55 \pm 1.20
Nymph premolt period	16.90 \pm 2.79	16.75 \pm 2.52
Nymph molt period	10.20 \pm 1.35	9.95 \pm 1.42
Adult feeding period	9.12 \pm 1.42	9.04 \pm 1.84

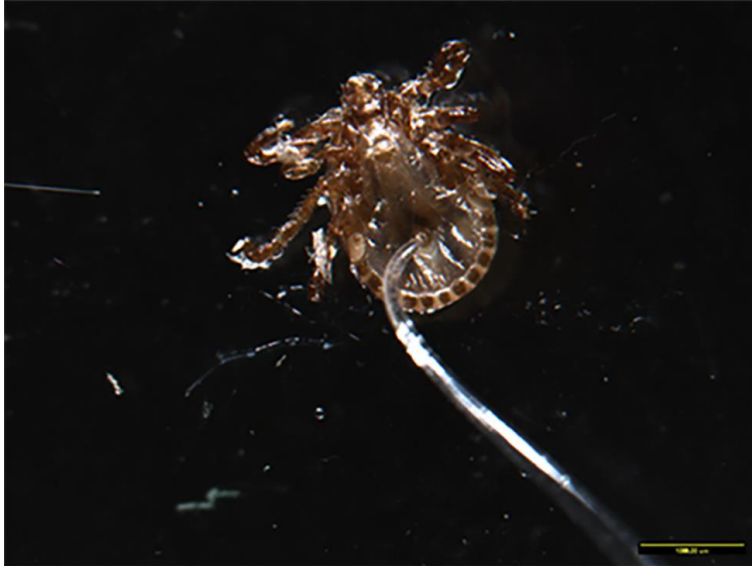
Technical Appendix Table 3. Numbers of mice for feeding in the life cycle of the *H. longicornis* ticks in the SFTSV study

Source	SFTSV group		Control group	
	Mice for feeding and detection (ticks/mouse)	Mice for feeding	Mice for feeding and detection (ticks/mouse)	Mice for feeding
Generation 1 adults	3 (4)	0	3 (4)	0
Eggs	0	0	0	0
Larvae	5 (50)	5	5 (50)	5
Nymphs	5 (10)	5	5 (10)	5
Generation 2 female adults	5 (5)	5	5 (5)	5
Generation 2 male adults	5 (5)	4	5 (5)	3

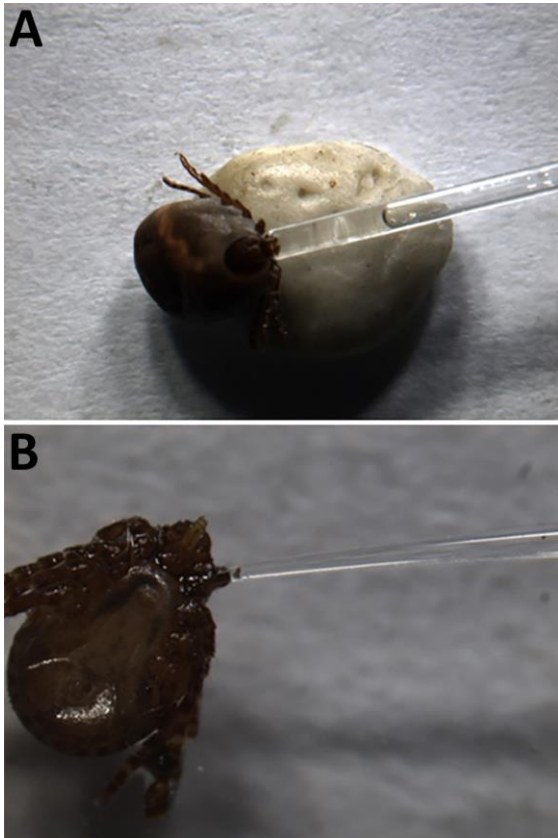


Technical Appendix Figure 1. Experimental framework of *H. longicornis* ticks microinjected with SFTSV.

We microinjected 45 adult *H. longicornis* ticks with SFTSV cell culture dilution (5.92×10^5 copies/mL) and microinjected 45 other adult ticks with PBS for negative control. Ticks were then fed on Balb/c mice. All control ticks were found to be SFTSV negative. To evaluate the SFTSV transmission from ticks to mice, we performed reverse transcription PCR and IFA of mice serum samples. To evaluate transovarial and transstadial transmission, we detected SFTSV RNA by PCR of 15 pools of eggs, 25 pools of larvae, 25 pools of nymphs, 25 males, and 25 females. We collected 4 pools of saliva and blood lymph from molted adults (generation 2) and used reverse transcription PCR assay for detection. We performed IFA of tissue smears of molted adults (generation 2) to show the localization of SFTSV. OT, oral transmission; TO, transovarial transmission; TT, transstadial transmission.



Technical Appendix Figure 2. Microinjection of adult *H. longicornis* ticks with SFTSV or PBS.



Technical Appendix Figure 3. Saliva and hemolymph collection from *H. longicornis* ticks. A) Collection of saliva from generation 2 adults. B) Collection of hemolymph from generation 2 adults.