Transmission potential of *Rickettsia felis* infection by *Anopheles gambiae* mosquitoes

Constentin Dieme[a,b,1], Yassina Bechah[a,1], Cristina Socolovschi[a], Gilles Audoly[a], Jean-Michel Berenger[a], Ousmane Faye[b], Didier Raoult[a], and Philippe Parola[a,2]

[a]Faculté de Médecine, Aix Marseille Université, Unité de Recherche sur les Maladies Infectieuses Tropicales et Emergentes, UM63, CNRS 7278, IRD 198, INSERM 1095, 13385 Marseille Cedex 5, France; and [b]Laboratoire d'Ecologie Vectorielle et Parasitaire, Université Cheikh Anta Diop de Dakar, BP 5005 Dakar, Senegal

Edited by Carolina Barillas-Mury, National Institutes of Health, Bethesda, MD, and approved May 1, 2015 (received for review July 21, 2014)

A growing number of recent reports have implicated *Rickettsia felis* as a human pathogen, paralleling the increasing detection of *R. felis* in arthropod hosts across the globe, primarily in fleas. Here *Anopheles gambiae* mosquitoes, the primary malarial vectors in sub-Saharan Africa, were fed with either blood meal infected with *R. felis* or infected cellular media administered in membrane feeding systems. In addition, a group of mosquitoes was fed on *R. felis*-infected BALB/c mice. The acquisition and persistence of *R. felis* in mosquitoes was demonstrated by quantitative PCR detection of the bacteria up to day 15 postinfection. *R. felis* was detected in mosquito feces up to day 14. Furthermore, *R. felis* was visualized by immunofluorescence in salivary glands, in and around the gut, and in the ovaries, although no vertical transmission was observed. *R. felis* was also found in the cotton used for sucrose feeding after the mosquitoes were fed infected blood. Natural bites from *R. felis*-infected *An. gambiae* were able to cause transient rickettsemias in mice, indicating that this mosquito species has the potential to be a vector of *R. felis* infection. This is particularly important given the recent report of high prevalence of *R. felis* infection in patients with "fever of unknown origin" in malaria-endemic areas.

Rickettsia felis | spotted fever | *Anopheles gambiae*

In 2002, *Rickettsia felis*, an obligate intracellular bacterium that belongs to the spotted fever group of *Rickettsia*, was definitively described (1, 2). Over the past 2 decades, an increasing number of reports have implicated *R. felis* as a human pathogen, paralleling an increase in reports of the detection of *R. felis* in arthropod hosts throughout the world (1, 3).

By 2011, more than 70 human cases of *R. felis* had been reported worldwide, including in Central and South America, Asia, northern Africa, and Europe (1). More cases have been published since then, including the first probable human cases in Australia (4). In sub-Saharan Africa, recent studies have challenged the importance of *R. felis* infection in patients with "fever of unknown origin," with this bacterium detected in up to 15% of such patients (5–7). In 2011, a potential *R. felis* primary infection, called "yaaf," was suspected in the case of an 8-mo-old girl in Senegal with polymorphous skin lesions similar to those seen in patients from Mexico (8). The epidemiologic and clinical picture of this emerging infection in Africa, including its potential vectors, is poorly understood, however.

Various arthropods, but primarily fleas, have been associated with *R. felis* (1, 3). More specifically, the cat flea *Ctenocephalides felis* is the arthropod in which *R. felis* has been most frequently detected. To date, it is the sole confirmed biological vector of *R. felis*, with both horizontal and vertical transmission making this flea a potential reservoir for this bacterium (9–11). However, in some countries where *R. felis* appears to be highly prevalent, such as Senegal, neither cat fleas nor other arthropods have been implicated in its transmission (12).

Mosquitoes are the most important vectors of infectious diseases in humans, with more than one-half of the global population at risk for exposure to mosquito-borne infections (13, 14). *Anopheles gambiae* is known to be the primary vector of malaria in Africa, whereas *Aedes albopictus* is a vector of dengue and chikungunya (15, 16). Interestingly, *Ae. albopictus* and *An. gambiae* mosquito cells support *R. felis* growth (1, 17). In 2012, *Ae. albopictus* from Gabon and *An. gambiae* molecular form S (the primary African malarial vector) from Ivory Coast tested positive for *R. felis* by species-specific real-time quantitative PCR (qPCR) (17, 18). More recently, several mosquito species from Senegal were found to harbor *R. felis*, including *Ae. luteocephalus*, *An. arabiensis*, *An. ziemanni*, *An. pharoensis*, *An. funestus*, and *Mansonia uniformis* (5). These data raise new issues with respect to the epidemiology of *R. felis* in Africa, including the degree of vector competence of mosquitoes. The objective of this work was to study the acquisition and transmission of *R. felis* by *An. gambiae* mosquitoes in an experimental model of infection.

Results

Infection of Mosquitoes by Membrane Feeding Systems. No significant difference in mortality was observed between the infected and control mosquitoes. In trial 1, a total of 92 complete mosquitoes were tested for *R. felis* acquisition and persistence, using *R. felis*-infected blood. In trial 2, using *R. felis*-infected XTC-cellular media, 45 were tested. Mosquitoes from trials 1 and 2 were positive for *R. felis* up to day 15 (D15) and D14 postinfection, respectively. The infection rate was 36% (33/92) for trial 1 and 93% (42/45) for trial 2 (Table S1). The infection rate for trial 2 was significantly greater than that of trial 1 ($P < 10^{-6}$).

The disseminated infection rate of *An. gambiae* females during the day (D) 0 to D15 postinfection in trial 1 is shown in Table 1. A total of 40 mosquitoes were dissected, and *R. felis* DNA was detected in ovaries and salivary glands up to D10 and D14 postinfection, respectively. The dissemination rate of *R. felis* in ovaries and salivary glands was 20%. *R. felis* was detected in the remaining body parts up to D15 postinfection, with a 42% infection rate. All of the controls tested negative. These results support the acquisition and dissemination of *R. felis* in mosquitoes.

Significance

***Rickettsia felis* is a ubiquitous, recently described human pathogen that has been identified as an important cause of unexplained fever in patients in sub-Saharan Africa. The epidemiology of *R. felis* infection, including its potential arthropod vectors, is poorly understood, however. The results of our experimental model of infection suggest that *Anopheles gambiae* mosquitoes, the primary malarial vectors in sub-Saharan Africa, have the potential to be vectors of *R. felis* infection.**

Author contributions: D.R. and P.P. designed research; C.D., Y.B., G.A., and J.-M.B. performed research; C.D., Y.B., C.S., O.F., and D.R. analyzed data; and C.D., Y.B., and P.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]C.D. and Y.B. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: philippe.parola@univ-amu.fr.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1413835112/-/DCSupplemental.

Table 1. qPCR analysis of *R. felis* dissemination in *An. gambiae* (ovaries and salivary glands) on different days following meals consisting of infected blood

DPI	No. tested	qPCR-positive samples, n (%)		
		Ovaries	Salivary glands	Body
D2	5	2 (40)	1 (20)	3 (60)
D4	5	3 (60)	3 (60)	4 (80)
D6	5	0	0	0
D8	5	1 (20)	1 (20)	4 (80)
D10	5	2 (40)	1 (20)	1 (20)
D12	5	0	1 (20)	2 (40)
D14	5	0	1 (20)	0
D15	5	0	0	3 (42)
Total	40	8 (20)	8 (20)	17 (42)

***R. felis* Transmission and Elimination by Feces.** In trial 1, DNA copies of *R. felis* were found on the cotton used for the sucrose feeding of infected mosquitoes between D4–D8 and D9–D15 postinfection. In trial 2, when the cotton had not been changed and instead was maintained from D1 to D14, the qPCR results were positive for *R. felis* DNA (Table 2). In addition, *R. felis* DNA was detected in mosquito feces up to 14 d postinfection in trial 2. At D8–D14 postinfection, the *R. felis* DNA copies were higher in the feces [cycle threshold (Ct) at 31.8 and 33.6 in qPCR1 and qPCR2, respectively) than at D1- D7 postinfection (Ct at 33.2 and 35.9 in qPCR1 and qPCR2, respectively). F1 larvae, nymphs, and unfed female and male adults from both trials were examined in groups of 10 for vertically transmitted *R. felis* infection by qPCR amplification. *R. felis* was not detected in any F1 progeny of the infected or control adults. In conclusion, after acquisition by mosquitoes, *R. felis* has been demonstrated to be eliminated in saliva (that is contained in the cotton used for sucrose feeding) and the feces, but transovarial transmission has not been demonstrated.

Mouse Model. In the preliminary experiment (experiment 1), *R. felis* was detected at D1 in the blood of inoculated mice (qPCR1; mean Ct, 28.3 ± 1.8), but not in the controls. *R. felis* was also detected in all of the mosquitoes tested immediately after their blood meal (day 0) and 1 d after feeding on mice inoculated with *R. felis* (Table 3). The number of positive samples decreased on days 2 and 5 ($P = 0.20$ and 0.007, respectively), and bacterial DNA was not detected in the mosquitoes tested at D6 ($P = 0.004$), D14 ($P = 0.01$), and D15 ($P = 0.0005$) (Table 3). At D16 postfeeding, roughly 20% of tested mosquitoes were positive for *R. felis.* This positive sample had lower Ct, corresponding to higher DNA copies, relative to the Ct found at D0, D1, and D2 (Table 3).

At 14 d after feeding on the blood of infected mice, mosquitoes ($n = 150$) were fed on two healthy mice. Subsequently, the blood of these mice was tested daily by qPCR for the presence of *R. felis*. A bacteremia (Ct, 32 in qPCR1 and 33 in qPCR3) was observed in one-half of the mice, which appeared 2 d after the feeding and disappeared thereafter (Table 4).

In experiments 2–5, a bacteremia (mean Ct, 26.3 ± 2.1; $n = 8$ in qPCR1) was observed in all of the inoculated mice on D1 after i.p. inoculation. Control mice tested negative. The blood of the mice that served as the uninfected meal source for mosquitoes 12–14 d after these mosquitoes had been allowed to feed on the blood of infected mice was tested for the presence of *R. felis* using qPCR. In experiment 2, the mouse that had been used as the uninfected meal source tested negative. The mouse of experiment 4 tested positive for *R. felis* at D2, using both qPCR1 and qPCR3 (Ct, 32.5 and 31, respectively). In each of experiments 3 and 5, two mice were used. The results of qPCR1 and qPCR3 showed that one (experiment 3) and two (experiment 5) of these mice had been infected through mosquito bites (Table 4). Following this, *R. felis* was detected in 21–66% of the mosquitoes. No bacterial DNA was detected in the mosquitoes that fed on the control animals (Table 4). In summary, natural bites from *R. felis*-infected *An. gambiae* were able to cause transient rickettsemias in several mice in separate experimental models.

Localization of *R. felis* in Mosquitoes by Immunofluorescence Assay. Specific punctate staining revealed the presence of *R. felis* in salivary glands, but not adjacent tissues, on D1 postinfection (Fig. 1). In addition, more extensive *R. felis* staining was seen on D15 postinfection (Fig. 1). *R. felis* was also visualized in select ovarian compartments on D8 postinfection and in gravid females on D15 postinfection (Fig. 1). The most significant signal was detected in and around the midgut on D14 postinfection (Fig. 1). In contrast, immunofluorescence assay (IFA) using preimmune sera on sections from the same mosquito did not reveal a specific *R. felis* signal, and the control mosquitoes fed with uninfected blood exhibited no *R. felis* staining in the gut or other tissues. The orientation of the mosquitoes during a microscopic examination was facilitated by DAPI staining and green autofluorescence of certain tissues and chitin-rich regions with 488-nm excitation. The autofluorescence intensity was much lower in the far-red region of the spectrum and permitted ready detection of *Rickettsia* with a secondary antibody coupled to Alexa Fluor 647.

Discussion

The vectorial capacity of an arthropod is related to its ability to maintain disease transmission in the open environment. This term encompasses many factors underlying an arthropod's role in pathogen transmission, including vector abundance, host specificity, vector longevity, time for the microorganism to develop its life cycle within the vector, and environmental, ecological, behavioral, cellular, biochemical, and molecular factors (19). The vector competence of a mosquito is a subcomponent of vectorial capacity and corresponds to the intrinsic ability of the mosquito to become infected with, allow replication of, and subsequently transmit a pathogen to a susceptible host (14). In our experiments, we addressed only the vector competence of *An. gambiae* for *R. felis*.

Using both artificial and mouse feeding, the acquisition and persistence of *R. felis* infection was shown by detecting and visualizing the bacteria up to D15 postinfection in mosquitoes and up to D14 in mosquito feces. One of the strategies for most mosquito-borne pathogens to maintain viability is to exit the midgut as quickly as possible, to escape the peritrophic matrix (14). In this study, dissemination of the pathogen in mosquitoes was demonstrated by detection and visualization of *R. felis* from the midgut (hemocoel), ovaries, and salivary glands after ingestion of the infectious meal. The additional viability and replication of *R. felis* organisms within mosquitoes was suggested by its high titer in one positive mosquito at D16 postinfection relative to D0. In our study, *R. felis* dissemination within the mosquitoes was very rapid and concomitant with the colonization of salivary glands and ovaries. Along with being detected and visualized in the salivary glands, *R. felis* was also found in the cotton used for sucrose feeding and mosquito saliva.

Table 2. qPCR analyses of *R. felis* transmission by *An. gambiae* through cotton used for sucrose feeding, after meals consisting of infected blood or infected cellular media

Trial	DPI*	Positive/no. cotton (%)	Ct in qPCR1	Ct in qPCR2
Infected with blood (trial 1)	D1–D3	0	0	0
	D4–D8	1/8 (12.5)	34.7	35.9
	D9–D15	1/5 (20)	35.5	36.6
Infected with cellular media (trial 2)	D1–D14	1/2 (50)	32.5	35.5

*Cotton was maintained during that period, and then removed to be tested.

Table 3. Detection of *R. felis* DNA by qPCR1 and qPCR3 in mosquitoes that were allowed to feed on mice inoculated with 10^6 *R. felis* using the i.p. route

	Day postfeeding							
	D0	D1	D2	D5	D6	D14	D15	D16
No. positive/no. tested mosquitoes	6/6	6/6	4/5	1/6	0/4	0/3	0/7	1/5
Mean ± SD Ct value, qPCR1	32.6 ± 0.5	31.8 ± 3.5	33.2 ± 3.3	31.7	0	0	0	23.5
Mean ± SD Ct value, qPCR3	31.8 ± 2.3	31.5 ± 1.2	32 ± 5.8	30.9	0	0	0	21.9

The final volume of extracted DNA was 50 μL, and qPCR was performed with 5 μL of this extract.

This tissue dissemination of *R. felis* relates to the dissemination of other *Rickettsia* in other arthropod vectors. For example, many tick-borne rickettsial human pathogens, such as *Rickettsia slovaca, Rickettsia massiliae*, and *Rickettsia conorii*, multiply in almost all of the organs and fluids of its tick host, particularly in the ovaries and salivary glands. This may enable the transmission of rickettsiae transovarially and during feeding on animals, including humans (20–22). Viable *R. slovaca* also has been found in the feces of its tick vectors (21).

Excretion of viable rickettsiae in the feces of experimentally infected arthropods has been reported with *Rickettsia prowazekii*, the agent of epidemic typhus, in its vector the human body louse. Eliminated rickettsiae remained viable in feces, constituting a persistent source of infection. When scratching the bite site, the direct inoculation of infected feces is allowed into the abrasions (23). This pathway is also the main route of the transmission of *Rickettsia typhi*, the agent of murine typhus transmitted mainly by the rat flea *Xenopsylla cheopis* (24). The transmission by fleas of *R. felis* has been associated with the presence of the bacteria in salivary glands and excretion in the feces (10).

Here, although *R. felis* was detected and visualized in the ovaries of *An. gambiae* mosquitoes, vertical transmission was not observed. However, when the transovarial transmission of a microorganism exists in its associated arthropod, the rate of transmission is not necessarily 100%. In various models of mosquito/viral transmission, low transovarial transmission rates have been observed in mosquito offspring (25, 26). This low transmission is also the case for tick–rickettsia associations, with a variation in the percentage of infected eggs obtained from the females of the same tick species infected with the same rickettsial strain. The reasons for this remain unknown (27). In our study, the lack of detection of *R. felis* in mosquito offspring may be explained by the fact that not all progeny were tested, rather than the complete absence of vertical transmission. Furthermore, the recent detection of *R. felis* in an *An. arabiensis* male collected in the field in Senegal suggests vertical transmission of *R. felis* in mosquitoes, considering that male mosquitoes do not consume blood, but rather feed on nectar and plant juices (5).

The ability of mosquitoes to transmit bacterial diseases to humans is poorly understood. Bacteria considered nonpathogenic endosymbionts, including *Wolbachia* spp., have been associated with mosquitoes (28, 29). Mosquitoes are also considered mechanical vectors of *Francisella tularensis* causing tularemia in humans, and the possibility of transmission by adult mosquitoes that have acquired the pathogen from their aquatic larval habitats has been recently suggested (30). Because *R. felis* infects mosquitoes, we studied the ability of these insects to transmit the bacteria using mice as hosts. We found that *R. felis* was transmitted to healthy mice through the bite of infected mosquitoes, a result that demonstrates the horizontal transmission of *R. felis* through mosquitoes in our model. These results suggest that mosquitoes may play a role in the epidemiologic cycle of *R. felis*.

An. gambiae species have a large area of distribution in Africa, ranging from the sub-Saharan region to central African forests to southern Africa (31). Because of its anthropophilic nature and high density, *An. gambiae* is considered the most important vector of human malaria in Africa (32, 33). A recent study comparing the epidemiology of *R. felis* infection and malaria in northern and sub-Saharan Africa found a significant linear trend of increasing risk for *R. felis* infection. The risks were lowest in regions where *An. gambiae* was absent or rare (e.g., France, Tunisia, Algeria), unlike in rural Senegal (5). A higher incidence of *R. felis* was observed during the rainy season in the subtropics, a period coinciding with the presence and activity of *Anopheles* mosquitoes (5).

We still do not fully understand the transmission cycle of *R. felis*. The range of vertebrate species serving as *R. felis* reservoir hosts is largely unknown, although the role of dogs as potential reservoir hosts has been suggested (34, 35). *R. felis* has been detected in the blood of patients with fever of unknown origin, as well as in asymptomatic persons in Africa (5–7), raising questions about the role of humans as *R. felis* reservoirs.

Artificial models are increasingly used to demonstrate vector competence, although it is difficult to definitively conclude that an arthropod is a vector of human disease, given that experimental models cannot be conducted in humans. It is important to follow these studies with those that approximate natural conditions. In addition, identification of other wild or domestic mosquito species that function as vectors is needed for a better understanding of the modes of transmission. Nonetheless, a high prevalence of *R. felis* infection in patients with fever of unknown origin in malaria-endemic areas and the detection of *R. felis* in mosquitoes in the field in these areas have been documented. Alongside the present results, which indicate that *An. gambiae* (a mosquito that readily bites humans) has the potential to be a vector of *R. felis* infection, these important arguments raise the question whether *An. gambiae* could be transmitting *R. felis* infections to humans living in malaria-endemic regions.

Table 4. Detection of *R. felis* by qPCR in the blood of healthy mice on which *An. gambiae* mosquitoes were allowed to feed 14 d (experiments 1–3) or 12 d (experiments 4 and 5) after having been allowed to feed on mice inoculated by *R. felis*

		Ct obtained by qPCR in blood mice (qPCR1/qPCR3)*			No. mosquito specimens that fed on mice/no. qPCR positive (%)
Experiment (E)	Mouse (M)	D1	D2	D3	
E1	M1	N	32/33	N	ND
	M2	N	N	N	ND
E2	M1	N	N	N	18/27 (66)
E3	M1	30/32	N	N	14/43 (32)**
	M2	N	N	N	
E4	M1	N	32.5/31	N	12/49 (24)
E5	M1	33/34	N	N	14/43 (32)
	M2	N	35 /34	N	9/41 (21)

*The final volume of extracted DNA was 50 μL, and qPCR was performed with 5 μL of this extract. N, negative; ND, not done.

**In E3, M1 and M2 were exposed together simultaneously to 43 mosquitoes.

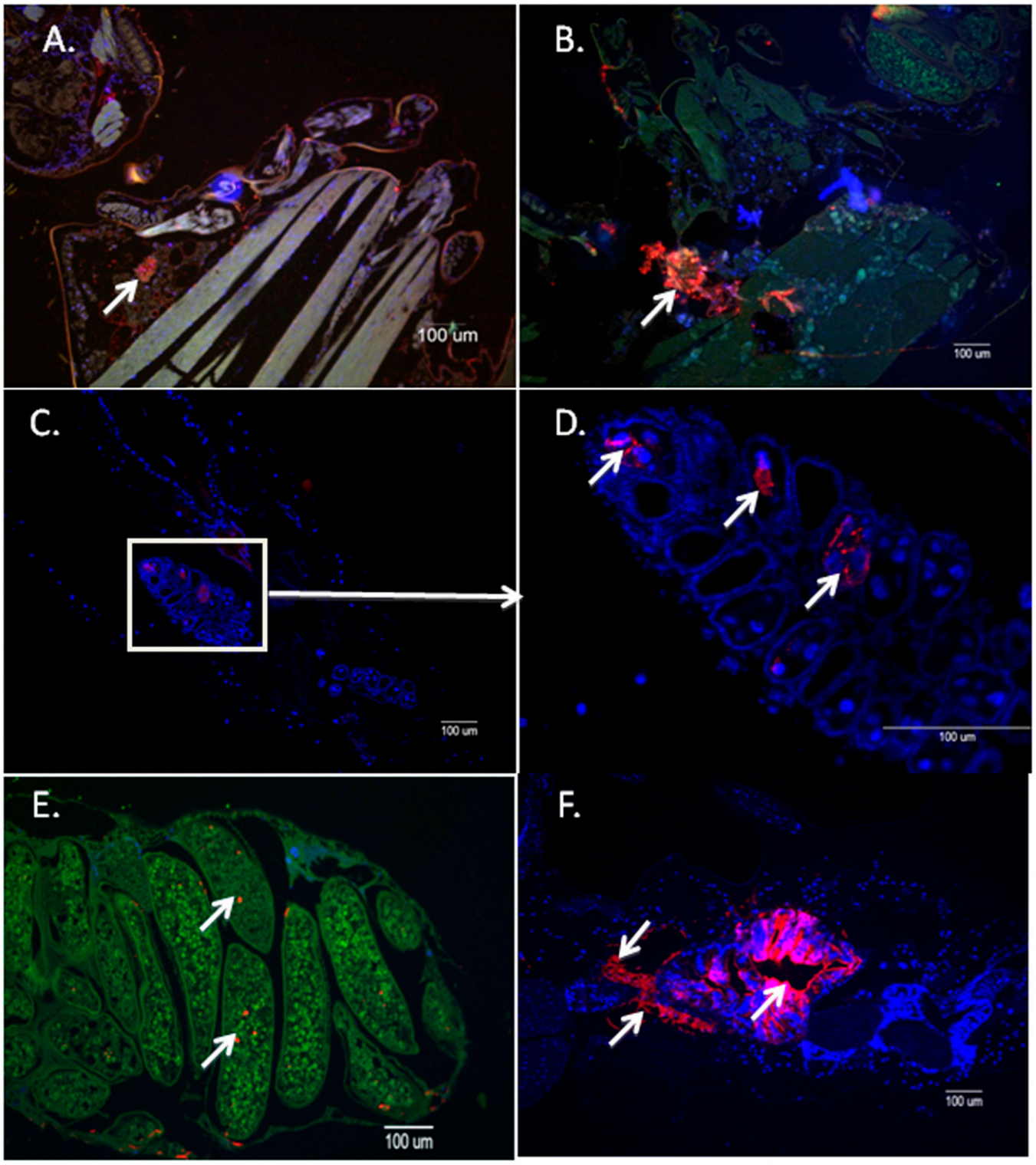

Fig. 1. Rickettsial dissemination in *R. felis*-infected mosquitoes over 15 DPI, days postinfection. (*A*) 1 DPI: *R. felis* in salivary glands. (*B*) 15 DPI: *R. felis* in salivary glands; (*C* and *D*) 8 DPI: *R. felis* in ovaries. (*E*) 15 DPI: *R. felis* in a gravid female. (*F*) 14 DPI: *R. felis* in and around the midgut.

Materials and Methods

Mosquitoes and Bacteria. Mosquitoes were reared in our laboratory using standard methods at 26 ± 1 °C, a relative humidity of 70–90%, and a 12-h photoperiod in stand-alone incubators (Panasonic cooled incubator) (36). Adults were fed at will with defibrinated sheep's blood using Hemotek membrane feeding systems and cotton impregnated with a 10% sucrose solution (37). The larvae were reared until the nymph stage in trays containing distilled water up to 1.5 cm in depth. The nymphs were collected daily and transferred to a mosquito cage (Bug Dorm 1; Bioquip Products) (38). *R. felis* strain Marseille-URRWFXCal$_2^T$ (2) was used for all of the experiments and this strain was isolated and maintained in XTC-2 cells, as described previously (39). Bacterial viability was assessed using the LIVE/DEAD BacLight Kit (Molecular Probes). For the artificial infection model, the titer of the meal used for the infection was ~10^7 PFU/mL and consisted of

infected cellular media or 400 μL of bacterial suspension, 6 mL of defibrinated sheep's blood, and 5 mM ATP, as described previously (40).

Membrane Feeding System Experimentation. Oral infections were performed as described previously with minor changes (41). Three- to 5-d-old female mosquitoes were sucrose-starved for 3 h before being allowed to feed for 45 min on *R. felis*-infected blood (trial 1) or *R. felis*-infected XTC-cellular media (trial 2) at 30 °C. The blood meals and infected cellular media were administered in aluminum membrane feeding systems with a collagen membrane. The engorged mosquitoes were collected with a modified bioquip vacuum (AC/DC Aspirator) and placed in 8″ × 8″ × 8″ (20.3 cm) metal cages (Bioquip Products). The mosquitoes were incubated as described above and were fed at will with a 10% sucrose solution in cotton (1 cm square × 0.8 cm thick).

A total of 205 mosquitoes were fed *R. felis*-infected blood and 144 were fed uninfected blood (controls) on D0. These females were then fed uninfected blood at D4, D7, D10, and D13 postinfection (trial 1). For trial 2, 112 mosquitoes were fed *R. felis*-infected XTC cellular media and 57 were fed uninfected XTC (control) on D0. All of the infected and control females were then separately fed uninfected blood on D1 and D7 postinfection.

Each day, both the infected and the control mosquitoes in trials 1 and 2 that had died naturally were tested for the presence of *R. felis*. In addition, for trial 1 on D0 and D15, five and nine mosquitoes, respectively, were killed and tested for *R. felis* acquisition and infection. For trial 2, three and two mosquitoes were killed on D0 and D14, respectively, and tested for *R. felis* acquisition and infection. Each mosquito was processed separately.

To measure *R. felis* dissemination and transmission at different days postinfection, five mosquitoes were also killed every 2 d, and their ovaries and salivary glands were dissected (trial 1) and tested by qPCR.

The cotton used for the sucrose solution was changed on D3, D8, and D15 postinfection for trial 1 and was maintained from D1 to D14 for trial 2. Each day, the cotton was moistened with the 10% sucrose solution via pipette. To recover the feces of the infected and control mosquitoes, filter paper of the same size of the base of the cages was introduced, and fecal spots were collected when the paper was changed every 7 d. To determine the presence of *R. felis*, the cotton used for the infected and control mosquitoes or the filter paper used for the infected and control fecal spots was eluted separately in 210 μL of Buffer G2 for 24 h. Then 200 μL of each elution was used for DNA extraction, followed by centrifugation for 30 s, pressure applied with a piston, and qPCR (17). In both trials, eggs from the mosquitoes exposed to *R. felis*-infected and uninfected food were collected, and the larvae were reared at 26 ± 1 °C, 70–90% relative humidity, and a 12-h photoperiod. For each trial and group, first-generation larvae, nymphs, and female and male adult mosquitoes were pooled in sets of 10, and the presence of *R. felis* was determined by qPCR.

Mouse Model. The following experimental protocol was approved by the Institutional Animal Care and Use Committee of Aix-Marseille Université. The mice were handled according to the rules of Décret no. 8 87–848 (October 19, 1987, Paris). The animals were housed in a dedicated facility and fed sterile food and water ad libitum. The mice were also observed daily for any signs of discomfort or distress. Mosquito feeding was performed on anesthetized mice using ketamine/xylazine, and all efforts were made to minimize suffering.

Seven week-old female BALB/c mice were obtained from Charles River Laboratories. For each experiment, each animal was inoculated with 10^6 *R. felis* using the i.p. route. In addition, for each experiment, one group of BALB/c mice was inoculated with PBS and served as the control group. To confirm the infection of mice, blood was sampled at D1 postinoculation, by retro-orbital puncture after ketamine/xylazine anesthesia, and tested by qPCR as described below. In a preliminary experiment (experiment 1), at D1 after *R. felis* inoculation, two mice were anesthetized via the i.p. route. A total of 426 mosquitoes were allowed to feed on these mice, and 300 mosquitoes were allowed to feed on one uninfected mouse control. After 1 h of feeding, mosquito samples (n = 3–7) were collected at different time points, and the presence of *R. felis* within these samples was tested by qPCR. The remainder of the gorged mosquitoes was fed on cotton impregnated with a 10% sucrose solution. On D14 after blood feeding, these mosquitoes were allowed to feed on two healthy mice. Then, from D0 to D4, mouse blood (200 μL) was tested daily for the presence of *R. felis* using qPCR, as described below.

In four additional successive experiments (experiments 2–5), 27–84 mosquitoes that were allowed to feed on an inoculated mice at D1 postinoculation were then allowed to feed on one healthy mouse (experiments 2 and 4) or two healthy mice (experiments 3 and 5), 12 or 14 d later. From D1 to D4, mouse blood (200 μL) was tested daily for the presence of *R. felis* using qPCR, as described below. All of the mosquitoes were then tested by qPCR to obtain their ratio of infection by *R. felis*.

Detection of *R. felis* by qPCR Amplification. DNA was extracted from whole mosquitoes, mosquito organs, mosquito progeny, cotton and feces, and from the blood of mice using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. Each PCR was performed with a CFX96 qPCR Detection System (Bio-Rad), using 5 μL of DNA in 15 μL of Master Mix under previously described conditions (17). The negative controls included DNA extracted from the ovaries, salivary glands, remaining body parts, and progeny of uninfected mosquitoes, as well as from the blood meal, fecal spots, and cotton from uninfected mosquitoes. *R. felis* DNA served as a positive control.

All of the samples were screened for the presence of *R. felis* with the following primers: Rfel_phosp_MBF, 5′-GCAAACATCGGTGAAATTGA-3′; Rfel_phosp_MBR, 5′-GCCACTGTGCTTCACAAACA-3′; and Rfel_phosp_MBP, 6FAM-CCGCTTCGTTATCCGTGGGACC, which target a phosphatase gene (qPCR1). The specificity of qPCR was tested in silico and on the 31 *Rickettsia* spp. from our laboratory. For qPCR1, the Ct values of 35, 32.5, 29, 26.9, and 23.6 correspond to 10^3, 10^4, 10^5, 10^6, and 10^7 copies of DNA, respectively. The samples were considered positive when the qPCR1 Ct was <36 and when samples also tested positive using another qPCR system, a standard procedure in our laboratory (17).

The mosquitoes infected by the membrane system were also tested by qPCR2 using the following primers: Rfel_ guano_MBF, 5′GCATATACTTTATTGTGCGCAAGTT-3′; Rfel_guano_MBR, 5′-TTTATCGATTGACAGAAGAAGAAATCA-3′; and Rfel_guano_MBP, 6FAM-TCGCTTTTTGGGATTGTTTGCCAGA, which target a guanosine polyphosphate gene (qPCR2) (42). For the animal model, in addition to the qPCR1 system, the presence of *R. felis* was confirmed by qPCR3, using specific primers and probes F (5′-CCCTTTTCGTAACGCTTTGCT-3′), R (5′-GGGCTAAACCAGGGAAACCT-3′) and P (6-FAM-TGTTCCGGTTTTAACGGCAGATACCCA-TAMR), which target a fragment of the *R. felis Orf* B gene (17). For the qPCR2 and qPCR3 confirmation tests, the samples are considered positive at a Ct of <37 (42).

IFA. A total of 14 female mosquitoes were killed and tested by IFA for the presence of *R. felis*. For trial 1, these mosquitoes included four, three, and three infected mosquitoes on D1, D8, and D15, respectively, and two infected and two control mosquitoes on D14 for trial 2. IFA was performed on 3-μm-thick paraffin-embedded sections from formalin-fixed mosquitoes using a Ventana Benchmark autostainer. After deparaffinization, each tissue section was fixed in 4% paraformaldhyde/PBS for 1 h at room temperature. IFA was performed as described previously with minor changes (*SI Materials and Methods*) (43). Sections from infected mosquitoes stained with preimmune rabbit serum and sections of control mosquitoes stained with polyclonal anti-*R. felis* served as negative controls.

Statistical Analysis. The presence of *R. felis* DNA in mosquitoes, expressed as the number of positive specimens in qPCR, was compared among the different groups using the χ^2 test or Fisher exact test. Data were considered significant at $P < 0.05$. Statistical analyses were performed using OpenEpi version 3.0.1.

ACKNOWLEDGMENTS. We thank Didier Fontenille for providing *An. gambiae* eggs, Hubert Lepidi for thick paraffin-embedded mosquito sections, and Nathalie Duclos, Marion Le Bideau, and Celine Perreal for *R. felis* strain Marseille-URRWFXCal$_2^T$.

1. Parola P (2011) *Rickettsia felis*: From a rare disease in the USA to a common cause of fever in sub-Saharan Africa. *Clin Microbiol Infect* 17(7):996–1000.
2. La Scola B, et al. (2002) Emended description of *Rickettsia felis* (Bouyer et al. 2001), a temperature-dependent cultured bacterium. *Int J Syst Evol Microbiol* 52(Pt 6):2035–2041.
3. Reif KE, Macaluso KR (2009) Ecology of *Rickettsia felis*: A review. *J Med Entomol* 46(4): 723–736.
4. Williams M, Izzard L, Graves SR, Stenos J, Kelly JJ (2011) First probable Australian cases of human infection with *Rickettsia felis* (cat-flea typhus). *Med J Aust* 194(1):41–43.
5. Mediannikov O, et al. (2013) Common epidemiology of *Rickettsia felis* infection and malaria, Africa. *Emerg Infect Dis* 19(11):1775–1783.
6. Richards AL, et al. (2010) Human infection with *Rickettsia felis*, Kenya. *Emerg Infect Dis* 16(7):1081–1086.
7. Socolovschi C, et al. (2010) *Rickettsia felis*-associated uneruptive fever, Senegal. *Emerg Infect Dis* 16(7):1140–1142.
8. Mediannikov O, et al. (2013) Description of "yaaf," the vesicular fever caused by acute *Rickettsia felis* infection in Senegal. *J Infect* 66(6):536–540.
9. Hirunkanokpun S, Thepparit C, Foil LD, Macaluso KR (2011) Horizontal transmission of *Rickettsia felis* between cat fleas, *Ctenocephalides felis*. *Mol Ecol* 20(21):4577–4586.
10. Thepparit C, Hirunkanokpun S, Popov VL, Foil LD, Macaluso KR (2013) Dissemination of bloodmeal acquired *Rickettsia felis* in cat fleas, *Ctenocephalides felis*. *Parasit Vectors* 6(1):149.

11. Wedincamp J, Jr, Foil LD (2002) Vertical transmission of *Rickettsia felis* in the cat flea (*Ctenocephalides felis* Bouché). *J Vector Ecol* 27(1):96–101.
12. Roucher C, Mediannikov O, Diatta G, Trape JF, Raoult D (2012) A new *Rickettsia* species found in fleas collected from human dwellings and from domestic cats and dogs in Senegal. *Vector Borne Zoonotic Dis* 12(5):360–365.
13. Adler PH, Tuten HC, Nelder MP (2011) Arthropods of medicoveterinary importance in zoos. *Annu Rev Entomol* 56:123–142.
14. Beerntsen BT, James AA, Christensen BM (2000) Genetics of mosquito vector competence. *Microbiol Mol Biol Rev* 64(1):115–137.
15. Bonizzoni M, Gasperi G, Chen X, James AA (2013) The invasive mosquito species *Aedes albopictus*: Current knowledge and future perspectives. *Trends Parasitol* 29(9): 460–468.
16. Cohuet A, Harris C, Robert V, Fontenille D (2010) Evolutionary forces on *Anopheles*: What makes a malaria vector? *Trends Parasitol* 26(3):130–136.
17. Socolovschi C, Pagés F, Raoult D (2012) *Rickettsia felis* in *Aedes albopictus* mosquitoes, Libreville, Gabon. *Emerg Infect Dis* 18(10):1687–1689.
18. Socolovschi C, Pages F, Ndiath MO, Ratmanov P, Raoult D (2012) *Rickettsia* species in African *Anopheles* mosquitoes. *PLoS ONE* 7(10):e48254.
19. Beaty BJ, Marquardt WC (1996) *The Biology of Disease Vectors* (Univ Press of Colorado, Niwot, CO), pp 51–72.
20. Matsumoto K, Ogawa M, Brouqui P, Raoult D, Parola P (2005) Transmission of *Rickettsia massiliae* in the tick, *Rhipicephalus turanicus*. *Med Vet Entomol* 19(3):263–270.
21. Rehacek J (1984) *Rickettsia slovaca*, the organism and its ecology. *Acta Sci Nat Brno* 18:1–50.
22. Socolovschi C, et al. (2012) Why are there so few *Rickettsia conorii conorii*-infected *Rhipicephalus sanguineus* ticks in the wild? *PLoS Negl Trop Dis* 6(6):e1697.
23. Houhamdi L, Fournier PE, Fang R, Lepidi H, Raoult D (2002) An experimental model of human body louse infection with *Rickettsia prowazekii*. *J Infect Dis* 186(11): 1639–1646.
24. Azad AF (1990) Epidemiology of murine typhus. *Annu Rev Entomol* 35:553–569.
25. Lambrechts L, Scott TW, Gubler DJ (2010) Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Negl Trop Dis* 4(5):e646.
26. Nikolay B, Diallo M, Faye O, Boye CS, Sall AA (2012) Vector competence of *Culex neavei* (Diptera: Culicidae) for Usutu virus. *Am J Trop Med Hyg* 86(6):993–996.
27. Burgdorfer W, Varma MG (1967) Trans-stadial and transovarial development of disease agents in arthropods. *Annu Rev Entomol* 12:347–376.
28. Rainey SM, Shah P, Kohl A, Dietrich I (2014) Understanding the *Wolbachia*-mediated inhibition of arboviruses in mosquitoes: Progress and challenges. *J Gen Virol* 95(Pt 3): 517–530.
29. Ricci I, et al. (2012) Mosquito/microbiota interactions: From complex relationships to biotechnological perspectives. *Curr Opin Microbiol* 15(3):278–284.
30. Bäckman S, Näslund J, Forsman M, Thelaus J (2015) Transmission of tularemia from a water source by transstadial maintenance in a mosquito vector. *Sci Rep* 5:7793.
31. Sinka ME, et al. (2012) A global map of dominant malaria vectors. *Parasit Vectors* 5:69.
32. Verhulst NO, Smallegange RC, Takken W (2012) Mosquitoes as potential bridge vectors of malaria parasites from non-human primates to humans. *Front Physiol* 3:197.
33. Ngom HM, et al. (2013) Spatio-temporal analysis of host preferences and feeding patterns of malaria vectors in the sylvo-pastoral area of Senegal: Impact of landscape classes. *Parasit Vectors* 6(1):332.
34. Hii SF, et al. (2011) Molecular evidence of *Rickettsia felis* infection in dogs from Northern Territory, Australia. *Parasit Vectors* 4:198.
35. Parola P, et al. (2013) Update on tick-borne rickettsioses around the world: A geographic approach. *Clin Microbiol Rev* 26(4):657–702.
36. Awono-Ambene HP, Diawara L, Robert V (2001) Comparison of direct and membrane feeding methods to infect *Anopheles arabiensis* with *Plasmodium falciparum*. *Am J Trop Med Hyg* 64(1-2):32–34.
37. Wong PS, Li MZ, Chong CS, Ng LC, Tan CH (2013) *Aedes* (Stegomyia) *albopictus* (Skuse): A potential vector of Zika virus in Singapore. *PLoS Negl Trop Dis* 7(8):e2348.
38. Moutailler S, Bouloy M, Failloux AB (2007) Short report: Efficient oral infection of *Culex pipiens quinquefasciatus* by Rift Valley fever virus using a cotton stick support. *Am J Trop Med Hyg* 76(5):827–829.
39. Raoult D, et al. (2001) A flea-associated *Rickettsia* pathogenic for humans. *Emerg Infect Dis* 7(1):73–81.
40. Kilpatrick AM, Fonseca DM, Ebel GD, Reddy MR, Kramer LD (2010) Spatial and temporal variation in vector competence of *Culex pipiens* and *Cx. restuans* mosquitoes for West Nile virus. *Am J Trop Med Hyg* 83(3):607–613.
41. Diallo M, et al. (2005) Potential role of sylvatic and domestic African mosquito species in dengue emergence. *Am J Trop Med Hyg* 73(2):445–449.
42. Dieme C, et al. (2015) *Rickettsia* and *Bartonella* species in fleas from Reunion Island. *Am J Trop Med Hyg* 92(3):617–619.
43. Audoly G, et al. (2011) Effect of rickettsial toxin VapC on its eukaryotic host. *PLoS ONE* 6(10):e26528.