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Alain Le Coupanec, Divya Babin, Michèle Bouloy, Valérie Choumet

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Short communication

Clone 13-infected *Aedes aegypti* salivary components inhibit Rift Valley fever virus pathogenicity

Alain Le Coupanec [a], Divya Babin [a], Michèle Bouloy [a], Valérie Choumet [a,b,*]

[a] *Unité de Génétique Moléculaire des Bunyavirus, Institut Pasteur, 25 rue du Dr Roux, Paris, France*
[b] *Unité Interactions Moléculaires Flavivirus-Hôtes, Institut Pasteur, 25 rue du Dr Roux, Paris, France*

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Abstract

Rift Valley fever virus (RVFV) continues to cause large outbreaks among humans and domestic animals in Africa. RVFV Clone 13, a naturally attenuated clone, is a promising vaccine which was used during the 2009–2010 outbreak in South Africa and played a key role in the control of the disease. In this work, we infected *Aedes aegypti* mosquitoes with RVFV Clone 13 and prepared salivary gland extracts (SGE). C57BL/6-NRJ male mice were infected with a mixture of SGE infected by Clone 13 and the ZH548 RVFV strain. With the injection of increasing doses of Clone 13-infected SGE, all mice were protected. Our results suggest Clone 13 infected SGE contain unique antiviral components able to counteract the replication of RVFV when injected into vertebrates.

Keywords: *Aedes aegypti*; Arbovirus; Rift Valley fever virus; Clone 13; Salivary gland; Pathogenicity

1. Introduction

Rift Valley fever virus (RVFV) is an arthropod-borne virus transmitted by mosquito vectors. This arbovirus was first isolated in Kenya in 1931 during epizootics in domestic animals [1]. Initially confined to Africa where periodic epidemics and epizootics occurred in sub-Saharan regions and in Egypt, RVFV has spread to the Middle East since 2000 and constitutes a risk to other geographic regions. RVFV is mainly transmitted to animals by mosquitoes, whereas humans are more often infected by close contact with sick animals [2]. Different mosquito species are involved in various geographic areas where the disease is endemic. RVFV can be maintained in an enzootic cycle involving *Aedes* mosquitoes such as *Ae. vexans* in West Africa [3]. The severity of RVFV zoonosis, as well as the capability to cause major epidemics in cattle and humans, has led to the listing of RVF a notifiable disease according to Council Directive 82/894/EEC EEC of 21 December 1982 on the notification of animal diseases and the virus is considered as a potential biological weapon.

RVFV is enveloped and spherical with a diameter of 80–120 nm. Belonging to the *Bunyaviridae* family, RVFV possesses a single stranded segmented RNA genome composed of large (L), medium (M), and small (S) segments [4]. The NSs protein encoded by the S segment was found to suppress induction of the antiviral type I interferon [5].

Clone 13 is a naturally attenuated variant that was cloned from strain of RVF virus (74HB59) isolated from a human case in the Central African Republic. It has been found to be avirulent in rodents. Mice or hamsters survive large infectious doses of up to 10^6 PFU/mL of RVFV Clone 13 without developing any signs of disease. It is defective in the synthesis of the NSs protein, the non-structural protein encoded by the S RNA segment. The NSs gene of Clone 13 contains, in addition to two conservative coding changes, a large internal deletion

* Corresponding author. Unité de Génétique Moléculaire des Bunyavirus, Institut Pasteur, 25 rue du Dr Roux, Paris, France. Tel.: +33 145688630; fax: +33 140613474.
E-mail address: vchoumet@pasteur.fr (V. Choumet).

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of 549 nucleotides that removes 69% of the open reading frame [6]. In addition, Clone 13 is highly immunogenic; mice receiving 10 pfu or more of Clone 13 achieved seroconversion and protection rates of at least 90% [6]. Moreover, a long lasting immunity was obtained. Clone 13 was tested as a vaccine candidate in sheep and cattle [7,8]. Since 2010, Clone 13 has been registered as a vaccine for animals (cattle, sheep and goats) and used in South Africa. More than 10 million RVF Clone 13 vaccine doses were used during the 2009–2010 RVF outbreak in South Africa, and played a key role in the control of the disease [9].

In this project, our objective was to test whether salivary gland extracts (SGE) of *Aedes aegypti* infected with Clone 13 were able to modulate the pathogenicity of RVFV. During a blood meal, blood-sucking insects are subject to defensive responses from the vertebrate. In this context, the mosquito injects saliva that plays multiple roles. Saliva proteins have angiogenic, anti-hemostatic, anti-inflammatory and immunomodulatory properties [10–13]. These various activities of saliva proteins toward the host immune response also affect disease transmission by the vector. Co-injection of arboviruses (La Crosse virus, Cache Valley virus, West Nile virus, Saint-Louis encephalitis virus, Western equine encephalitis virus) and mosquito (*Aedes triseriatus*, *Aedes aegypti*, *Culex pipiens*, *Culex tarsalis*) saliva or salivary gland extracts potentiates viral infection of the vertebrate [14]. In this study, we tested the effects of the salivary gland extracts from *Ae. aegypti* mosquitoes infected with Clone 13 on the pathogenicity of RVFV in mice.

2. Materials and methods

2.1. Ethics statement

All studies on animals followed the guidelines for the ethical use of animals from the European Communities Council Directive of November 24, 1986 (86/609/EEC). All animal experiments were approved and conducted in accordance with the Institut Pasteur Biosafety Committee. The study protocols were approved by the Comité d'Ethique for l'Expérimentation Animale (CEEA) – Ile de France – Paris – Comité 1.

2.2. Mice, cells and virus

We used 4 week-old male C57BL/6-NRJ mice for infections (Janvier, France).

Vero E6 and C6/36 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 10 μg/ml of penicillin and 10 U/ml of streptomycin.

In all experiments, the RVFV ZH548 strain was obtained from a cell culture of C6/36 cells, and the naturally avirulent Clone 13 strain was obtained from a cell culture of Vero E6 cells. The ZH548 strain was isolated from a febrile human during the 1977 RVF epidemic in Egypt and the Clone 13 strain was isolated from a nonfatal human case in Bangui (Central African Republic) in 1974 [6]. These viruses were produced under BSL3 conditions.

2.3. Mosquitoes

We used the Paea strain of *Ae. aegypti*, a laboratory colony initiated with mosquitoes collected in French Polynesia in 1994 and maintained in the laboratory for 400–450 generations. Dehydrated mosquito eggs were placed in water to hatch. Adult mosquitoes were reared in a room held at 25 ± 1 °C and 80% relative humidity, and having a light/dark ratio of 12 h/12 h. The larvae were fed on brewer's yeast tablets and adults were fed on sugared water (10%).

2.4. Mosquito infection by the Clone 13 RVFV strain

Twelve ml of rabbit blood was collected in heparinized tubes (2%). Red blood cells were separated from plasma by centrifugation for 15 min at 500 g, washed 3 times for 5 min at 500 g in 1× PBS and were resuspended in a volume of PBS allowing to recover the initial volume of blood. Five-day old female mosquitoes were placed in boxes sealed with veils and were fed for 15 min on 37 °C thermostated glass feeders covered with chicken skin and filled with a mixture containing 2 mL of red cells, 1 mL of Clone 13 virus solution (10^8 pfu/mL) and 30 μL of ATP (5×10^{-3} M). After bloodmeal, mosquitoes were anesthetized at 4 °C and blood-fed females were sorted and transferred into cardboard containers covered with mosquito nets. Mosquitoes were dissected at various time points after oral exposure. Salivary glands and midguts were removed, placed in 300 μl of lysis buffer and kept frozen at −80 °C until RNA extraction (Nucleospin RNA II kit, Macherey Nagel). Whole mosquitoes were also collected over time and their infectious status was determined by qRT-PCR after RNA extraction as described above.

2.5. Mosquito salivation

After the incubation period, Clone 13-exposed mosquitoes were anesthetized at 4 °C, legs and wings were removed and bodies were placed on a double-sided tape fixed on a glass slide. The proboscis was inserted manually into a 10 μL pipette low binding tip (Corning) filled with 5 μL of filtered PBS 1× or DMEM + Glutamax (Dulbecco) containing 2% FBS. The tip content was collected 45 min later in a tube containing 300 μL DMEM + Glutamax (Dulbecco) containing 2% FBS and the virus titer in the solution was determined by plaque assay. Saliva was collected at two time points: 14 and 16 days after exposure to the infectious blood-meal.

2.6. Virus titration by plaque assay on Vero E6 cells

RVFV-containing samples were titrated on Vero E6 cells by the plaque assay method [15]. E6 cells were grown in DMEM + Glutamax (Dulbecco) containing 10% decomplemented FBS, 10 U/mL penicillin and 10 μg/mL streptomycin in 6-well plates containing 10^6 cells per mL for plaque assays. Tenfold serial dilutions of each saliva sample to be titrated were prepared in DMEM medium that contained 2% FBS, 10 U/mL penicillin and 10 μg/mL streptomycin. 270 μL

of inoculum dilution was deposited in each well of a 6-well plate and incubated with for 1 h at 37 °C in a CO_2 incubator. Then, 4 mL of agar (culture medium containing 2% FBS and 2% agarose) were deposited in each well and cells were incubated for three days. The agar was removed and plaques were then revealed with a 0.2% solution of crystal violet containing 3.7% formaldehyde and 20% ethanol.

2.7. Titration of Clone 13 viral RNA in mosquito by qRT-PCR

Synthetic RNA transcripts for RVFV were generated to construct a standard curve. The targeted region in the RVFV sequence (nucleotides 33 to 3615 of the M protein gene) was amplified by PCR and ligated into the pCR II TOPO vector (Invitrogen). The plasmid was then linearized using EcoRI restriction enzyme and purified using QIAquick PCR purification kit (Qiagen). RNA transcripts were prepared *in vitro* using the RiboMAX™ Large Scale RNA Production Systems (Promega) appropriate for either SP6 or T7 RNA polymerase. Residual DNA was eliminated with several DNAse treatments (Turbo DNA-free (Ambion)). After quantification by spectrophotometer, RNA transcript solution was stored at −80 °C.

We used the Power SYBR Green RNA-to-Ct One-Step Kit (Applied Biosystems, Carlsbad, California) according to the manufacturer's protocol for qRT-PCR. To allow amplification of a 108 bp sequence located between nucleotide 1485 and nucleotide 1593 of the M segment of RVFV the primers were as follows: upper 5′-CATGGATTGGTTGTCCGATCA-3′ and lower 5′-TGAGTGTAATCTCGGTGGAAGGA-3′. Each mosquito sample was analyzed in duplicate against a standard curve produced from a specific concentration range of synthetic RNA. We amplified the samples on an Applied Biosystems 7500 instrument using the following PCR program: a reverse transcriptase (RT) step for 30 min at 50 °C; inactivation of the RT enzyme and activation of DNA polymerase for 10 min at 95 °C; 40 PCR cycles of 15 s at 95 °C and 1 min at 60 °C (annealing temperature of primers), during which fluorescence data is collected; and finally, 20 s at 95 °C with ramping 19 min 59 s for melting curves.

2.8. Preparation of salivary gland extracts (SGE) from mosquitoes exposed to Clone 13-infected artificial bloodmeal

Female mosquitoes were blood-fed five days after hatching. Two weeks later (which corresponded to the extrinsic incubation period of RVFV in *Ae. aegypti*), 100 salivary glands (SG) from Clone 13 blood-fed mosquitoes were dissected and placed in 100 µL 1× PBS. SG-containing tubes were stored at −80 °C. SGE were prepared by sonicating the SGs (five times at 4 min each with a pulse ratio of 2 s on/2 s off) and centrifuging the crude extract at 13,000 rpm for 15 min at 4 °C. The supernatant (SGE) was transferred to clean tubes and stored at −80 °C. The protein concentration was determined by spectrophotometry at 280 nm (Nanodrop) for each SGE preparation in order to assess the proper conduct of the protocol. The inoculums used in our experiments were equivalent to 1 µl of SGE (1 SG or 0.5 salivary gland pair), 2 µl of SGE (2 SG or 1 SG pair), 4 µl of SGE (4 SG or 2 SG pairs) and 8 µl of SGE (8 SG or 4 SG pairs).

2.9. Infection of mice with the ZH548 strain

Groups of 10 mice were anesthetized intraperitoneally with 50 µl of a ketamine/xylazine mixture consisting of 2 mL of 2% Rompun (Bayer), 4 ml of Imalgene 1000 (Merial), 4 ml of sterile water (Gibco) and 2 mL of PBS 1× (Gibco). "Pathogen-free" male mice C57BL/6NRj (Janvier) aged 5 weeks and weighing 15–20 g each, were infected in a BSL3 animal facility by the intradermal route in the absence or presence of increasing doses of RVFV Clone 13 SGE (one SG pair per inoculum = SGP). Mice were observed for 13 days; experiments were reproduced three times. Survival curves were analyzed using the Logrank and the Log-rank (Mantel–Cox) tests.

3. Results and discussion

3.1. Follow-up of Clone 13 virus infection in orally infected Ae. aegypti females by qRT-PCR

We followed the replication kinetics of Clone 13 in the mosquito midgut and SG after *Ae. aegypti* orally exposure. RNA copy numbers were measured by qRT-PCR at various times after oral exposure. In addition, we determined the level of viral particles in the saliva of mosquitoes (Fig. 1).

The RNA copy number reached a plateau in the midgut at day 6 (D6) post-infection (Fig. 1A). This was 7 days before high number of RNA copies was detected in the SG, with a peak happening at D15 (Fig. 1B). Moreover, all mosquitoes tested at various times after infection were found positive for the virus (Supplementary Fig. 1). Only 37% of the saliva samples tested at D14 were positive and titers were low (5–90 pfu/saliva) whereas 70% of those tested at D16 were positive with titers ranging between 25 and 2500 pfu/saliva (Fig. 1C). The extrinsic incubation period and the amount of viral particles in saliva are quite similar to those obtained with the ZH548 RVFV strain in the same mosquito strain with viral dissemination and transmission 16 days after exposure [16]. Our results are in agreement with Amraoui et al. [17], who observed similar rates of infection and extrinsic incubation period. For the following experiments, SGs were collected at D16 post-infection.

3.2. Effect of injection of Clone 13-infected SGE on RVFV pathogenecity in a mouse model

In this experiment, we investigated the role of Clone 13 SGE extracts on the pathogenicity of ZH548 RVFV in mice ($n = 10$). For this purpose, the RVFV ZH548 strain (10^2 pfu/mouse) was injected intradermally in the absence or in the presence of increasing doses of Clone 13 SGE (0.5–4 SGP equivalent per mouse). The results are presented in Fig. 2. We

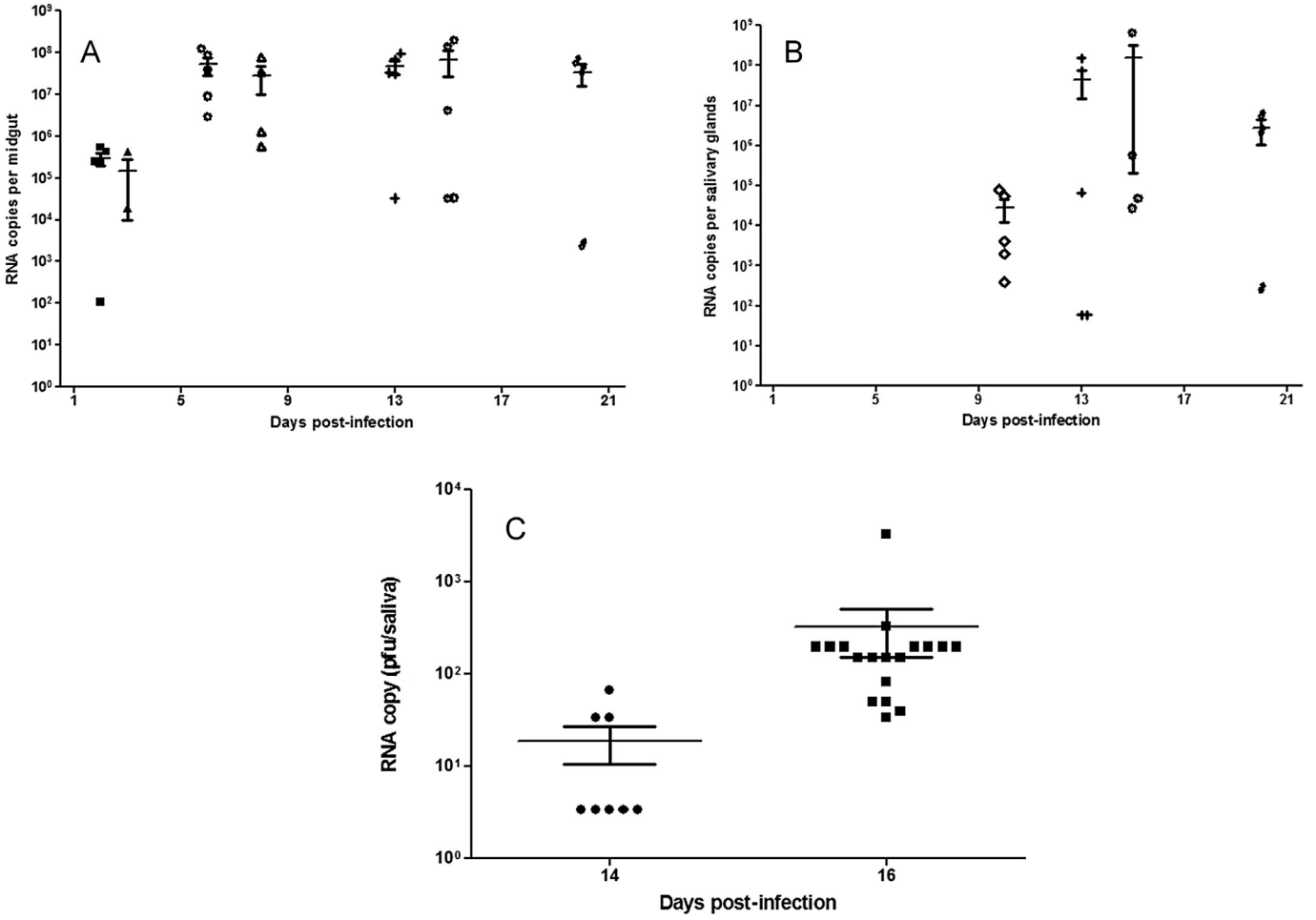

Fig. 1. RNA levels in midguts and SG, and viral particle levels in saliva of *Ae. aegypti* mosquitoes infected with Clone 13 virus. Mosquitoes were blood-fed on Clone 13-infected rabbit blood (10^8 pfu/ml). Midgut and SG of 5 mosquitoes per day post-infection (represented by different symbols according to the day of collect) were dissected in lysis buffer. RNA levels were determined by qRT-PCR. (A) Representation of the level of RNA copy in the midgut. The level of RNA was followed based on the days post-infection. (B) Representation of the level of RNA copy in the salivary glands based on the days post-infection. (C) This panel shows the level of viral particles contained in the saliva of mosquitoes infected by Clone 13 at 14 and 16 days post-infection. This level was measured by virus titration on Vero E6 cells. Only positive results are shown. Means and standard deviation for each sampling day are shown as bars and lines.

show that in the absence of Clone 13 SGE, all the mice died whereas in the presence of 0.5 or 1 Clone 13 SGP, the survival rate was 70%. Finally, with 2 and 4 Clone 13 SGP, the survival rate reached 100%.

Then we were interested to investigate whether the protective effect of Clone 13 SGE was still effective if the virus and 4 Clone 13 SGP were injected at the same time but at different sites on the mouse. Our results show that in the group injected with Clone 13 SGP at a distance from the site of injection of the virus, although the mortalities were delayed by 5 days, all mice had died by 13 days post-injection (Supplementary Fig. 2).

Three hypotheses might be raised to explain these observations. The first is that the protection is explained by the injection of high amounts of SGE. The second hypothesis is that the protection is due to the presence of Clone 13 at the site of injection of RVFV ZH548. The last hypothesis is that the presence of Clone 13 inside the SG may change the composition of salivary proteins which may result in the reduced pathogenicity of the wild-type virus.

To address the first hypothesis, we injected intradermally to 3 groups of 10 mice a mixture of the RVFV ZH548 strain with 4 non-infected SGP of *Ae. aegypti*. As shown in Fig. 3, although the mice survive longer (3 more days), all died at least 11 days after infection. This comparison proves that the protection of mice is explained by the presence of Clone 13 in the SG.

We wished to know if this protection was due to the activation of the interplay between Clone 13 and ZH548 with target cells in the mouse skin or to a modulation of the composition of the salivary proteins. If we consider the first hypothesis, two possibilities may explain the inhibition. We first tested whether Clone 13-infected SGE were still infectious after sonication and found no detectable virus by plaque assay. If the virus was killed by sonication, its remaining protein contents present in SG might still be able to compete with the wild-type virus for interaction with target cells. We therefore examined the effect of the co-injection of the Clone 13 strain, sonicated or not on RVFV pathogenicity in mice (Fig. 3). A partial protection was obtained in both cases. This

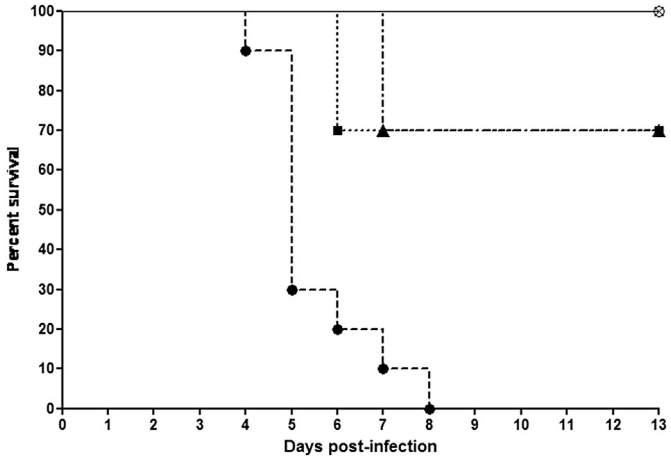

Fig. 2. Survival curves of C57BL/6-NRJ male mice after injection of 10^2 pfu/mouse of RVFV coupled with increasing doses of Clone 13-infected SGE. This experiment was performed with 10 mice per group. Curves with circle (full), square, triangle, plus and circle (empty) forms represent the mouse group that received respectively in addition to the dose of RVFV (10^2 pfu/mouse), 0, 0.5, 1, 2 and 4 Clone 13-infected SGP. Survival curves were analyzed using the Logrank and the Log-rank (Mantel–Cox) tests. They were found significantly different ($p < 0.001$).

observation suggests that Clone 13 may interact with target cells and compete with the wild-type virus, or is able to modify the bite site by changing the innate immune response. But the protection was not total as observed with infected SGE.

The last hypothesis was then considered. Both SGE and saliva from non-infected *Ae. aegypti* increased the pathogenicity of RVFV ZH548 strain in C57BL/6 NRJ mice [16]. Moreover, bites from RVFV-infected *Ae. aegypti* mosquitoes killed mice. Interestingly, Luplertlop et al. [18] have shown that Dengue virus increased in the SG the level of a peptide exhibiting anti-bacterial, anti-viral and anti-parasitic effects. In addition, a Cys-rich salivary secreted peptide was shown to restrict West Nile virus infection in *Culex* mosquito cells by activating the JAK-STAT pathway [19]. Homologues of this peptide exist in *Ae. aegypti* and *Ae. albopictus* SG. This result would imply that the Clone 13 strain may be able to trigger the expression of an antiviral response, a response that is not activated by ZH548 RVFV. Interestingly, several studies have shown that mutant RVFV with a truncated form of NSs did not replicate in the same way as the wild-type virus, in particular abortive infections were established in MRC-5 human fibroblasts [6]. Moreover, in *Ae. aegypti*, infection and transmission rates of a NSs deletion mutant virus were similar to wild type virus while dissemination rates were significantly reduced [20]. This study clearly showed a differential behavior of NSs deletion mutant in mosquitoes and suggested that immune antiviral response might occur in various organs such as salivary glands to impair viral replication. Indeed, it has been shown that Dengue virus activates in human keratinocytes the host genes involved in the antiviral immune responses such as intracellular RNA virus sensors Toll-Like Receptor-3, Retinoic Acid Inducible Gene-I, Melanoma Differentiation Associated gene-5 and the RNA-dependent protein kinase R [21]. The observed enhancement of WNV infection mediated by *Ae. aegypti* saliva was suggested to consist of a reduction of T lymphocyte and antiviral activity at the inoculation site, an elevated abundance of susceptible cell types, and a concomitant increase in immunoregulatory activity of IL-10 [22]. Since a protection against rather than an enhancement of RVFV infection is observed in our study, we propose that Clone 13 may trigger the synthesis of mosquito salivary proteins that would stimulate antiviral innate immunity in mice. These components may be able to counteract the replication of RVFV when injected into vertebrates. These antiviral components might also protect against other arboviral infections such as dengue, West Nile or chikungunya. Experiments are in progress to analyze the proteome of Clone 13 versus ZH548 infected *Aedes* SG in order to identify which proteins are modulated in the presence of each virus. In chikungunya virus infected *Ae. aegypti*, the differentially regulated salivary proteins were involved in virus survival, replication and transmission, suggesting a subversion of the insect cell metabolism by arboviruses [23]. Proteins involved in blood-feeding were proposed to favor virus transmission by exerting an increased anti-inflammatory effect. We can therefore propose that these proteins might be down-regulated in the salivary glands of Clone 13 infected *Ae. aegypti*.

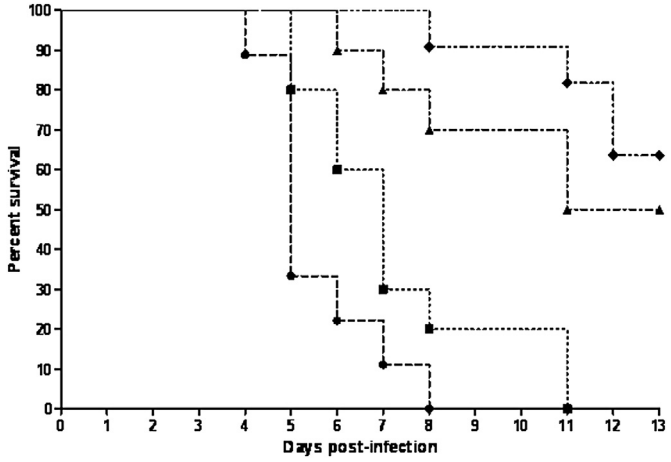

Fig. 3. Survival curves of C57BL/6-NRJ male mice injected with RVFV in the presence or absence of SGP or Clone 13 (inactivated or not). These experiments were performed with 10 mice per group. Curves with round, square, triangle and diamond forms represent the mouse group that received respectively in addition to the dose of RVFV (10^2 pfu/mouse), 0, 4 non-infected SGP, 10^6 pfu/mouse sonicated Clone 13 (inactivated) or 10^6 pfu/mouse active Clone 13.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2014.01.008.

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