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T. N. J. J. Nepomichene, F. N. Raharimalala, S. F. Andriamandimby, J.-P. Ravalohery, Anna-Bella Failloux, et al.. Vector competence of *Culex antennatus* and *Anopheles coustani* mosquitoes for Rift Valley fever virus in Madagascar.. *Medical and Veterinary Entomology*, 2018, 10.1111/mve.12291 .
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Submitted on 4 Apr 2018

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Vector competence of *Culex antennatus* and *Anopheles coustani* mosquitoes to Rift Valley Fever Virus in Madagascar

Running head: Vector competence of VRVF in Madagascar

Thiery Nirina Jean José Nepomichene^{1,2n}, Fara Nantenaina Raharimalala^{1n*}, Soa Fy Andriamandimby³, Jean-Pierre Ravalohery³, Anna-Bella Failloux⁴, Jean-Michel Heraud^{#3}, Sébastien Boyer^{#1,5}

Email: jthiery@pasteur.mg

Email: rfaranantenaina@pasteur.mg

Email: soafy@pasteur.mg

Email: jpierre@pasteur.mg

Email: anna-bella.failloux@pasteur.fr

Email: jmheraud@pasteur.mg

Email: sboyer@pasteur-kh.org

* Corresponding author: Fara Nantenaina Raharimalala, Institut Pasteur de Madagascar, Unit of Medical Entomology, Ambatofotsikely, 101-Antananarivo, Madagascar. + 261 20 22 401 64. rfaranantenaina@pasteur.mg

¹ Unit of Medical Entomology, Institut Pasteur de Madagascar

² Ecole Doctorale Science de la Vie et de l'Environnement, Université d'Antananarivo, BP 906 Antananarivo, Madagascar

³ Unit of Virology, Institut Pasteur de Madagascar, Ambatofotsikely, 101-Antananarivo, Madagascar

26 ⁴ Department of Virology, Arboviruses and Insect Vectors, Institut Pasteur, 25–28 rue du Dr
27 Roux, 75724 Paris, cedex 15, France

28 ⁵ Medical Entomology Platform, Institut Pasteur du Cambodge, 5 Bd Monivong, Phnom
29 Penh, Cambodia

30 [¶] These authors contributed equally to this work.

31 [#] These authors contributed equally to this work (co-last authors)

32 **Abstract**

33 *Culex antennatus* (Diptera: Culicidae), *Anopheles coustani* (Diptera: Culicidae) and
34 *Anopheles squamosus/cydippis* (Diptera: Culicidae) were found infected with Rift Valley
35 Fever virus (RVFV) during an epidemic that occurred in 2008-2009 in Madagascar. To
36 understand the role played by *Cx. antennatus* and *An. coustani* in maintenance and
37 transmission, we assessed RVFV vector competence of these two species. Mosquito body
38 parts and saliva of mosquitoes that fed on RVFV-infected blood were tested for RVFV using
39 real-time (RT-PCR) assays. Overall, we detected viral RNA virus in body parts and saliva at 5
40 days post infection (dpi) for both species. At 5 dpi, infection rates were 12.5% (3/24) and
41 15.8% (6/38), disseminated infection rates were 100% (3/3) and 100% (6/6), transmission rate
42 were 33.3% (1/3) and 83.3% (5/6), and transmission efficiencies were 4.2% (1/24) and 13.2%
43 (5/38) respectively for *Cx. antennatus* and *An. coustani*. Although RVFV detected in saliva
44 did not propagate onto Vero cells, these results support a potential role of these two-mosquito
45 species in the transmission of RVFV.

46

47 **Key-words:** Risk assessment, Arboviruses, Emergence, Madagascar

48

49 Introduction

50 Rift Valley Fever Virus (RVFV) belongs to the *Phlebovirus* genus and *Bunyaviridae* family.
51 It can cause severe and fatal illness in domestic animals (Woods et al. 2002). Humans can
52 develop an encephalitic, ocular or hemorrhagic syndrome (Kahlon et al. 2010). RVFV can be
53 transmitted by close contact with infectious tissues or through mosquito infectious bites
54 (Smithburn et al. 1948). Its geographical distribution has extended and it could emerge in an
55 area previously not known to have RVFV transmission (Balenghien et al. 2013).
56 In Madagascar, RVFV was detected for the first time in 1979, from a pool of multi and
57 monospecific mosquitoes caught in a rainy forest of the central eastern part of the island
58 (Fontenille et al. 1988). The monospecific pool was composed of *Mansonia uniformis*
59 (Diptera: Culicidae). Surprisingly, the virus was not detected neither in human nor in animals
60 (Clerc & P 1981). Since this first detection, RVFV reemerged in 1990-91 and 2008-09,
61 resulting in outbreaks that affected both animals and humans (Morvan et al. 1992; Fontenille
62 et al. 1989; Andriamandimby et al. 2010). Furthermore, during the last epidemic, RVFV was
63 detected in three mosquito species: *Culex antennatus*, *Anopheles coustani* and *Anopheles*
64 *squamosus/cydippis* (Ratovonjato et al. 2011). These mosquito species were also part of the
65 pool of mosquitoes examined in 1979 (Clerc & P 1981).
66 Twenty-four Malagasy mosquito species are potentially associated with RVFV transmission
67 in Madagascar (Tantely et al. 2015). These species belong to the genera of *Aedes*, *Anopheles*,
68 *Culex*, *Eretmapodites*, and *Mansonia*. All these mosquitoes feed preferentially on domestic
69 animals (cattle, sheep, and goat) but can also have opportunistic anthropophilic behavior
70 (Tantely et al. 2015).
71 Despite a recurrent circulation of RVFV (Andriamandimby et al. 2010; Ratovonjato et al.
72 2011; Gray et al. 2015) and the abundance of potential vectors (Tantely et al. 2015), no study
73 on vector competence has been carried out to date in Madagascar. To better understand the

74 mechanism of maintenance and transmission of RVFV in Madagascar, we performed a study
75 aiming to evaluate the vector competence of *Cx. antennatus* and *An. coustani*, two mosquito
76 species implicated in the last RVFV outbreak in Madagascar.

77

78 **Materials and Methods**

79 **Mosquito collection and rearing**

80 Engorged and gravid females of *Cx. antennatus* and *An. coustani* were captured in three
81 different sites of Madagascar where RVFV has been detected in cattle and human during the
82 2008-09 outbreaks (Figure 1).

83 Mosquito females were either captured in stables early in the morning or in zebu pens using
84 oral aspirators. Trapping in zebu pens was conducted from 06:00 pm to 06:00 am for two
85 consecutive days. After capture, mosquitoes were kept alive in cages with free access to a 6%
86 sugar solution. Upon arrival to the laboratory, mosquitoes were identified morphologically
87 and then maintained separately in breeding cages according to species. After laying, eggs
88 were immersed in 1.5 cm depth of dechlorinated water. Immature stages were then separated
89 and reared until the adult stage. Emerged adults were placed in cages up to 300 individuals
90 per cage. First generations of females (F1) aged from 3 to 5 days were used for vector
91 competence experiments.

92 **Virus for experimental infections**

93 The virus strain used in this study was isolated from a RVFV-infected pool of mosquitoes
94 collected during the 2008-09 outbreak in Fianarantsoa (Ratovonjato et al. 2011). Viral stocks
95 were prepared after three passages of the isolate on Vero E6 cells. Stocks were produced in
96 12-well tissue culture plates maintained at 37°C. After 72h, the supernatant was collected and
97 kept at -80°C. Viral titer was estimated by plaque reduction assay on Vero cells. For all
98 assay, we used a viral titer of 2.25×10^8 plaque-forming units (pfu)/mL.

99 **Artificial infection of mosquitoes**

100 Artificial infection experiments were performed with the Hemotek membrane feeding system
101 (Hemotek Ltd, UK). An infectious blood meal was prepared according to the protocol of
102 Moutailler et al. (Moutailler et al. 2007). Briefly, the infectious blood meal consisted of 2/3 of
103 erythrocytes (2 mL) and 1/3 (1 mL) of a viral suspension with ATP used as a phagostimulant
104 at a final concentration of 5×10^{-3} M. Mosquitoes were presented with the opportunity to
105 blood feed for a maximum of 30 min. Fully engorged female mosquitoes were sorted on ice
106 and maintained at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, humidity: $80\% \pm 10\%$, photoperiod: 12 h/12 h and then
107 provided with 6 % sucrose solution for 14 days. Experimental infections were conducted in
108 the Biosafety Laboratory Level-3 at the virology unit and were repeated three times.

109 **Virus detection**

110 Viral detection in mosquitoes was conducted at 2, 5, 8 days post-infection (dpi) for *An.*
111 *coustani* and at 2, 5, 8 and 14 dpi for *Cx. antennatus*.
112 Viral detection was carried out for the midgut, head and thorax, saliva, legs and wings. Except
113 saliva, all other samples were ground with a TissueLyzer in 200 μL of medium MEM
114 containing 40% SVF. Then, 140 μL of saliva and 140 μL of homogenate obtained from other
115 mosquito tissues/organs were used to extract viral RNA using an extraction Kit Nucleospin®
116 Dx Virus (Macherey-Nagel, Mauritius). RNA was eluted in a final volume of 50 μL in H_2O
117 RNase Free. Presence of RVFV was tested by real-time quantitative RT-PCR (Bird et al.
118 2007). Primers amplified a fragment of 90 base pair (bp) of the L segment encoding for the
119 viral polymerase.

120 **Analyses for vector competence**

121 Four parameters were studied and analyzed at each dpi: (a) the infection rate (IR) indicating
122 the proportion of mosquitoes with infected midgut among tested mosquitoes; (b) the
123 disseminated infection rate (DIR), corresponding to the proportion of individuals with

infected head, thorax, legs or wings among mosquitoes with midgut infected; (c) the transmission rate (TR) showing the proportion of individuals with infectious saliva among the mosquito with disseminated infection (i.e. individuals with infected head, thorax, legs or wings); and finally (d) the transmission efficiency (TE), defined as the proportion of individuals with infectious saliva among the total number of mosquitoes tested (Chouin-Carneiro et al. 2016).

To test if RVFV detected in mosquito saliva can propagate in cells as a proxy of the presence of infectious virions in the saliva, 100 μ L of saliva were inoculated on Vero E6 cells. RVFV from viral stocks were also inoculated as positive control. If a cytopathic effect was observed on cells after inoculation, then the saliva was considered as infectious containing replicating viral particles. The extrinsic incubation period (EIP) corresponding to the duration between the infectious blood meal and the first detection of the virus in mosquito saliva was estimated for each mosquito species.

Results

Among the 216 *Cx. antennatus* individuals fully engorged, 83 (38. 4%) were tested for the presence of virus in the midgut. The remaining 133 mosquitoes died before the period of testing. Values of IR, DIR, TR and TE are presented in Table 1. RVFV was detected in the head, thorax, legs, wings and saliva at 5 dpi (Table 1). We began to detect the IR at 2 dpi with 40% (4/10) of individuals tested and DIR, TR and TE at 5 dpi. At 5 dpi, values of IR, DIR, TR and TE were 12.5% (3/24), 100% (3/3), 33.3% (1/3) and 4.2% (1/24) respectively. DIR reached 100% from 5 dpi i.e all individuals with infected midgut were also infected in head, thorax, legs and wings. Thus, for *Cx. antennatus*, EIP was estimated to be 5 days and 4.16% of individual tested had infected saliva.

For *An. coustani*, among 232 individuals fully engorged, 64 (27.6%) were tested for the presence of virus in midgut. The remaining 168 mosquitoes died before the period of testing. Values of IR, DIR, TR and TE are presented in Table 2. RVFV was detected in head, thorax, legs, wings and saliva at 5 dpi (Table 2). IR values have been beginning detected at 2 dpi with 15% (3/20) of individuals tested and DIR, TR and TE at 5 dpi. At 5 dpi, values of IR, DIR, TR and TE were 15.8% (6/38), 100% (6/6), 83.3 (5/6) and 13.2% (5/38) respectively. DIR reached 100% from 5 dpi i.e all individuals with infected midgut had virus detected in head, thorax, legs and wings. Thus for *An. coustani*, EIP was estimated to be 5 days and 13.2% of individual tested had infected saliva.

From both *Cx. antennatus* and *An. coustani*, RVFV detected from saliva using real-time quantitative RT-PCR did not propagate onto Vero cell.

Discussion

Anopheles coustani and *Culex antennatus* collected in Madagascar were tested for their vector competence to RVFV. Results showed that 4.2% and 13.2% of *Cx. antennatus* and *An. coustani* respectively, were susceptible to RVFV infection with viral RNA detected in saliva. These results were consistent with descriptions of *An. coustani* and *Cx. antennatus* infected with RVFV in natural mosquito populations (Hanafi 2011, Ratovonjatovo 2011, Seufi & Galal 2010). Most importantly, we detected RVFV in saliva of both species which is a crucial information in vector competence assays as it shows that mosquitoes could transmit the virus to vertebrates during feeding.

The virus detected in saliva did not propagate onto Vero cells. These results could be explained by the sensitivity of our cell system that required a higher titer of virus in the saliva. Experimental infections of vertebrate (rodent) with infected mosquitoes is needed to demonstrate that viral particles detected in saliva are indeed infectious.

172 In our experiments, the EIP were 5 days for both *An. coustani* and *Cx. antennatus* which is
173 consistent with previous work from Turell et al. In this study, authors observed an EIP for *Cx.*
174 *antennatus* ranging from 3 to 10 dpi according titers of RVFV used (Turell et al. 2008).
175 Nevertheless, while they estimated the transmission efficiency at 84% (Turell et al. 2008), in
176 our case, using a similar viral titer of 10^8 pfu/mL, we obtained a transmission efficiency of
177 only 4.2%. This difference could be explained by the difference of viral strain used, genetic
178 background of mosquito population, and mode of infection (oral vs. intrathoracic inoculation)
179 (Turell et al. 2008).

180 Although *An. coustani* species have been detected positive for RVFV in nature, we showed
181 for the first-time evidence of replication of RVFV in salivary glands of *Anopheles* species.
182 Indeed, previous studies on *An. pharoensis* from Egypt and *An. stephensi* from laboratory
183 strain (US Army Bioengineering Research and Development Laboratory) never observed
184 release of RVFV in the saliva (Turell et al. 1996; Turell & Romoser 1994).

185 These findings highlight the association of *An. coustani* and *Cx. antennatus* with RVFV in
186 nature. This association between *An. coustani* and *Cx. antennatus* and some vertebrate hosts
187 of RVFV has been underlined in different regions of Madagascar. Indeed, these species were
188 found mostly zoophilic especially *Cx. antennatus* and attracted by goat, sheep and zebu in two
189 regions geographically distant in Madagascar (Nepomichene et al. 2015). It has also been
190 shown that *An. coustani* rest in stables during the day and analyses of ingested blood meal
191 showed that this species has a trophic preference for zebu. Nonetheless, this species was also
192 found to feed on human (Nepomichene, Tata, et al. 2015).

193 To conclude, *An. coustani* and *Cx. antennatus* were previously found associated with RVFV
194 in nature in Madagascar and in other countries. The vector competence described herein by
195 the four indices showed that the two species are susceptible to RVFV and can potentially
196 release RVFV during blood meal. These findings coupled with the trophic preference for

197 human and domestic animal strengthen the hypothesis of the role of these two mosquitoes'
198 species in the transmission of RVFV in Madagascar although only viral RNA and not
199 infectious viral particles were detected in saliva. This information is critical for implementing
200 vector control to prevent reemergence of RVFV.
201

Acknowledgements

We are grateful to the all staff form the Institut Pasteur de Madagascar that helped to conduct the study. The authors also thanks to Ms. Marie Vazeille and to Dr. Camillo Arias-Goeta, Department of Virology, Arboviruses and Insect Vectors, Institut Pasteur de Paris for their help to train staff on vector competence study. We also thank Dr. Marie Chrystine Solofoharivelo of Institut Pasteur de Madagascar and Dr. Thomas Walker of London School of Hygiene & Tropical Medicine, Department of Diseases Control. Keppel Street, London WC1E, United Kingdom for helping to edit this manuscript.

Authors'contributions

FNR, JMH and SB coordinate the project and designed experiments. FNR and TNJJN collected and breed mosquitoes and conducted experimental infection of mosquitoes. JPR performed cell culture and virus titration. FNR, TNJJN, SFA, ABF, JMH, SB analyzed the results and wrote the paper. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the internal project of the Institut Pasteur de Madagascar received by FNR and a subsidy from ACIP-2013 project funding by the Institut Pasteur in Paris.

Author details

226 ¹Institut Pasteur de Madagascar, Unité d'Entomologie Médicale, Ambatofotsikely, 101-
227 Antananarivo, Madagascar. ² Institut Pasteur de Madagascar, Virology Unit, National
228 Reference Laboratory for Arboviruses, Ambatofotsikely, 101-Antananarivo, Madagascar.
229 ³Institut Pasteur de Paris, Génétique Moléculaire des Bunyaviridés, Institut Pasteur, 25–28
230 Rue du Dr. Roux, 75724 Paris CEDEX 15, France.
231

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297

298

299 **Table 1- Infection, dissemination and transmission of RVFV by *Culex antennatus*.**

300

Day post-infection	Infection Rate (IR)	Disseminated Infection Rate (DIR)	Transmission Rate (TR)	Transmission Efficiency (TE)
2	40% (4/10)	0% (0/4)	- (0/0)	0% (0/10)
5	12.5% (3/24)	100% (3/3)	33.3% (1/3)	4.2% (1/24)
8	14.3% (3/21)	100% (3/3)	66.6% (2/3)	9.5% (2/21)
14	33.3% (3/9)	100% (3/3)	100% (3/3)	33.3% (3/9)

301 IR, Infection rate, proportion of infected mosquitoes among tested ones; DIR, Disseminated
 302 Infection Rate, proportion of mosquitoes with disseminated infection (head, wings, legs
 303 infected) among infected mosquitoes; TR, Transmission Rate, proportion of mosquitoes with
 304 infected saliva among mosquitoes with disseminated infection; TE, Transmission Efficiency,
 305 proportion of mosquitoes with infected saliva among tested ones.

306

307

308 **Table 2- Infection, dissemination and transmission of RVFV by *Anopheles coustani*.**

Day post-infection	Infection Rate (IR)	Disseminated Infection Rate (DIR)	Transmission Rate (TR)	Transmission Efficiency (TE)
2	15% (3/20)	0% (0/3)	- (0/0)	0% (0/20)
5	15.8% (6/38)	100% (6/6)	83.3 (5/6)	13.2% (5/38)
8	50% (3/6)	100% (3/3)	100 (3/3)	50% (3/6)

309 IR, Infection rate, proportion of infected mosquitoes among tested ones; DIR, Disseminated
310 Infection Rate, proportion of mosquitoes with disseminated infection (head, wings, legs
311 infected) among infected mosquitoes; TR, Transmission Rate, proportion of mosquitoes with
312 infected saliva among mosquitoes with disseminated infection; TE, Transmission Efficiency,
313 proportion of mosquitoes with infected saliva among tested ones.

314

Figure Legend

Fig. 1- The three study sites in Madagascar. Red dots design the study sites. Engorged and gravid females of *Cx. antennatus* and *An. coustani* were captured in three different sites in Madagascar: in Moramanga (18°51'27.60"S; 48° 7'40.20"E) located in the Alaotra-Mangoro region, in Ankazobe (18°24'16.81"S; 47° 3'4.37"E), in the Analamanga region, and in Tsiroanomandidy (18°50'37.16"S; 46° 1'55.79"E), in the Bongolava region.

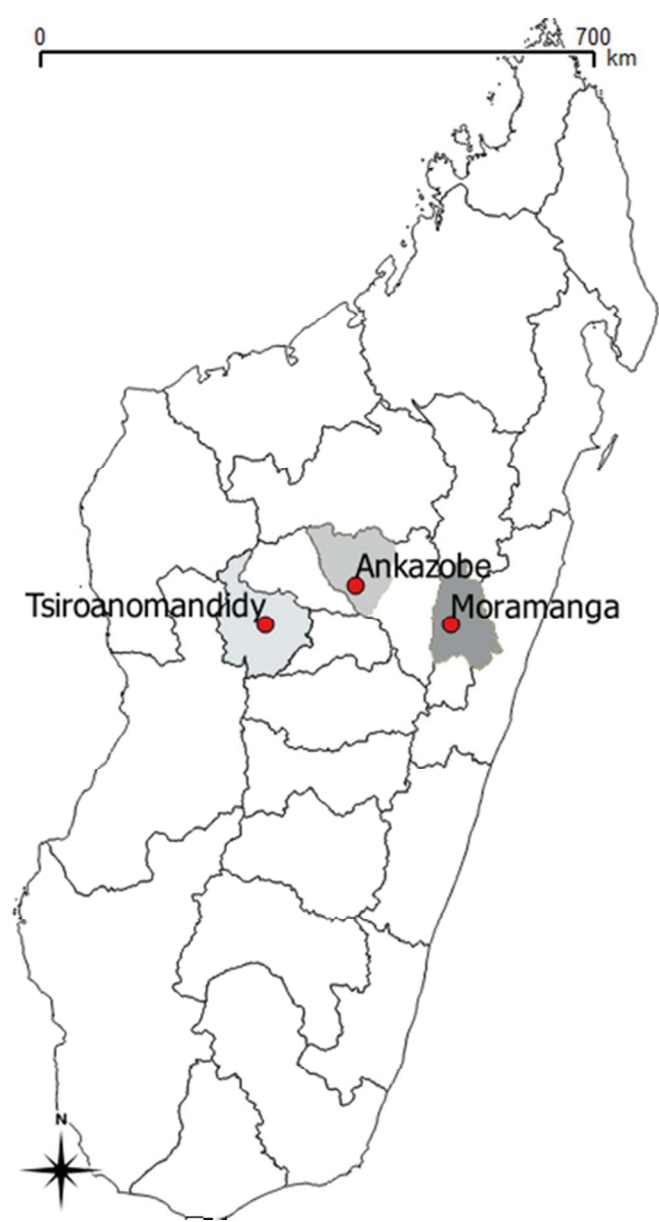


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41x73mm (300 x 300 DPI)