Vector competence of Culex antennatus and Anopheles coustani mosquitoes for Rift Valley fever virus in Madagascar.

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Running head: Vector competence of VRVF in Madagascar

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Abstract

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

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

Introduction

Rift Valley Fever Virus (RVFV) belongs to the *Phlebovirus* genus and *Bunyaviridae* family. It can cause severe and fatal illness in domestic animals (Woods et al. 2002). Humans can develop an encephalitic, ocular or hemorrhagic syndrome (Kahlon et al. 2010). RVFV can be transmitted by close contact with infectious tissues or through mosquito infectious bites (Smithburn et al. 1948). Its geographical distribution has extended and it could emerge in an area previously not known to have RVFV transmission (Balenghien et al. 2013).

In Madagascar, RVFV was detected for the first time in 1979, from a pool of multi and monospecific mosquitoes caught in a rainy forest of the central eastern part of the island (Fontenille et al. 1988). The monospecific pool was composed of *Mansonia uniformis (*Diptera: Culicidae). Surprisingly, the virus was not detected neither in human nor in animals (Clerc & P 1981). Since this first detection, RVFV reemerged in 1990-91 and 2008-09, resulting in outbreaks that affected both animals and humans (Morvan et al. 1992; Fontenille et al. 1989; Andriamandimby et al. 2010). Furthermore, during the last epidemic, RVFV was detected in three mosquito species: *Culex antennatus*, *Anopheles coustani* and *Anopheles squamosus/cydippis* (Ratovonjato et al. 2011). These mosquito species were also part of the pool of mosquitoes examined in 1979 (Clerc & P 1981).

Twenty-four Malagasy mosquito species are potentially associated with RVFV transmission in Madagascar (Tantely et al. 2015). These species belong to the genera of *Aedes, Anopheles, Culex, Eretmapodites*, and *Mansonia*. All these mosquitoes feed preferentially on domestic animals (cattle, sheep, and goat) but can also have opportunistic anthropophilic behavior (Tantely et al. 2015).

Despite a recurrent circulation of RVFV (Andriamandimby et al. 2010; Ratovonjato et al. 2011; Gray et al. 2015) and the abundance of potential vectors (Tantely et al. 2015) , no study on vector competence has been carried out to date in Madagascar. To better understand the

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

Materials and Methods

Mosquito collection and rearing

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

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

Virus for experimental infections

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

Artificial infection of mosquitoes

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

Virus detection

Viral detection in mosquitoes was conducted at 2, 5, 8 days post-infection (dpi) for *An. coustani* and at 2, 5, 8 and 14 dpi for *Cx. antennatus*.

Viral detection was carried out for the midgut, head and thorax, saliva, legs and wings. Except saliva, all other samples were ground with a TissuLyzer in 200 µL of medium MEM containing 40% SVF. Then, 140 µL of saliva and 140 µL of homogenate obtained from other mosquito tissues/organs were used to extract viral RNA using an extraction Kit Nucleospin® Dx Virus (Macherey-Nagel, Mauritius). RNA was eluted in a final volume of 50 µL in H_2O RNAse Free. Presence of RVFV was tested by real-time quantitative RT-PCR (Bird et al. 2007). Primers amplified a fragment of 90 base pair (bp) of the L segment encoding for the viral polymerase.

Analyses for vector competence

Four parameters were studied and analyzed at each dpi: (a) the infection rate (IR) indicating the proportion of mosquitoes with infected midgut among tested mosquitoes; (b) the 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 µL 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.

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

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

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

To conclude, *An. coustani* and *Cx. antennatus* were previously found associated with RVFV in nature in Madagascar and in other countries. The vector competence described herein by the four indices showed that the two species are susceptible to RVFV and can potentially 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

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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.

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References

Andriamandimby, S.F. et al., 2010. Rift valley fever during rainy seasons, Madagascar, 2008 and 2009. *Emerging Infectious Diseases*, 16(6), pp.963–970.

Balenghien, T. et al., 2013. Towards a better understanding of Rift Valley fever epidemiology in the south-west of the Indian Ocean. *Veterinary research*, 44, p.78. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3848763&tool=pmcentrez&rendertype=abstract [Accessed March 23, 2016].

Bird, B.H. et al., 2007. Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. *Journal of Clinical Microbiology*, 45(11), pp.3506–3513.

Chouin-Carneiro, T. et al., 2016. Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika Virus. *PLoS Neglected Tropical Diseases*, 10(3), pp.1–11. Available at: http://dx.doi.org/10.1371/journal.pntd.0004543.

Clerc, Y. & P, C., 1981. Rapport du Laboratoire des arbovirus 1980. *Arch Inst Pasteur Madag.*, 49, pp.65–69.

Fontenille, D., Mathiot, C. & Coulanges, P., 1988. [Hemorrhagic fever viruses in Madagascar]. *Archives de l'Institut Pasteur de Madagascar*, 54(1), pp.117–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3242423 [Accessed September 7, 2016].

Fontenille, D., Rakotoarivony, I. & Lepers, J.P., 1989. [Entomological results of the malaria program of the Pasteur Institute in the Malagasy Highland Plateaux in 1987-1988]. *Archives de l'Institut Pasteur de Madagascar*, 56(1), pp.275–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2576719 [Accessed July 6, 2016].

Gray, G.C. et al., 2015. Seroepidemiological Study of Interepidemic Rift Valley Fever Virus Infection Among Persons with Intense Ruminant Exposure in Madagascar and Kenya. *American Journal of Tropical Medicine and Hygiene*, 93(6), pp.1364–1370. Available

at: http://www.ajtmh.org/cgi/doi/10.4269/ajtmh.15-0383 [Accessed September 7, 2016].

Kahlon, S.S. et al., 2010. Severe Rift Valley Fever May Present with a Characteristic Clinical Syndrome. *American Journal of Tropical Medicine and Hygiene*, 82(3), pp.371–375.

Morvan, J. et al., 1992. First fatal human case of Rift Valley fever in Madagascar. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86(3), p.320. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1412665 [Accessed April 12, 2016].

Moutailler, S., Bouloy, M. & Failloux, A.-B.B., 2007. Short report: Efficient oral infection of Culex pipiens Quinquefasciatus by Rift Valley fever virus using a cotton stick support. *The American journal of tropical medicine and hygiene*, 76(5), pp.827–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17488899 [Accessed April 12, 2016].

Nepomichene, T.N.J.J., Elissa, N., et al., 2015. Species Diversity, Abundance, and Host Preferences of Mosquitoes (Diptera: Culicidae) in Two Different Ecotypes of Madagascar With Recent RVFV Transmission. *Journal of Medical Entomology*, 52(5), pp.962–969. Available at: http://jme.oxfordjournals.org/lookup/doi/10.1093/jme/tjv120 [Accessed September 7, 2016].

Nepomichene, T.N.J.J., Tata, E. & Boyer, S., 2015. Malaria case in Madagascar, probable implication of a new vector, Anopheles coustani. *Malaria journal*, 14(1), p.475. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4666205&tool=pmcentrez&rendertype=abstract.

Ratovonjato, J. et al., 2011. Detection, isolation, and genetic characterization of Rift Valley fever virus from Anopheles (Anopheles) coustani, Anopheles (Anopheles) squamosus, and Culex (Culex) antennatus of the Haute Matsiatra region, Madagascar. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*, 11(6), pp.753–759.

Seufi, A.M. & Galal, F.H., 2010. Role of Culex and Anopheles mosquito species as potential

vectors of rift valley fever virus in Sudan outbreak, 2007. *BMC Infectious Diseases*, 10(1), p.65. Available at: http://bmcinfectdis.biomedcentral.com/articles/10.1186/1471-2334-10-65.

Smithburn, K.C., Haddow, A.J. & Gillet, J.D., 1948. Rift Valley fever; isolation of the virus from wild mosquitoes. *British journal of experimental pathology*, 29(2), pp.107–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2077096&tool=pmcentrez&rendertype=abstract [Accessed April 12, 2016].

Tantely, L.M., Boyer, S. & Fontenille, D., 2015. Review article: A review of mosquitoes associated with Rift Valley fever virus in Madagascar. *American Journal of Tropical Medicine and Hygiene*, 92(4), pp.722–729.

Turell, M.J. et al., 2008. Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. *Journal of medical entomology*, 45(1), pp.102–108.

Woods, C.W. et al., 2002. An outbreak of Rift Valley fever in Northeastern Kenya, 1997-98. *Emerging infectious diseases*, 8(2), pp.138–44.

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

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)

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

315 **Figure Legend**

316

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

322

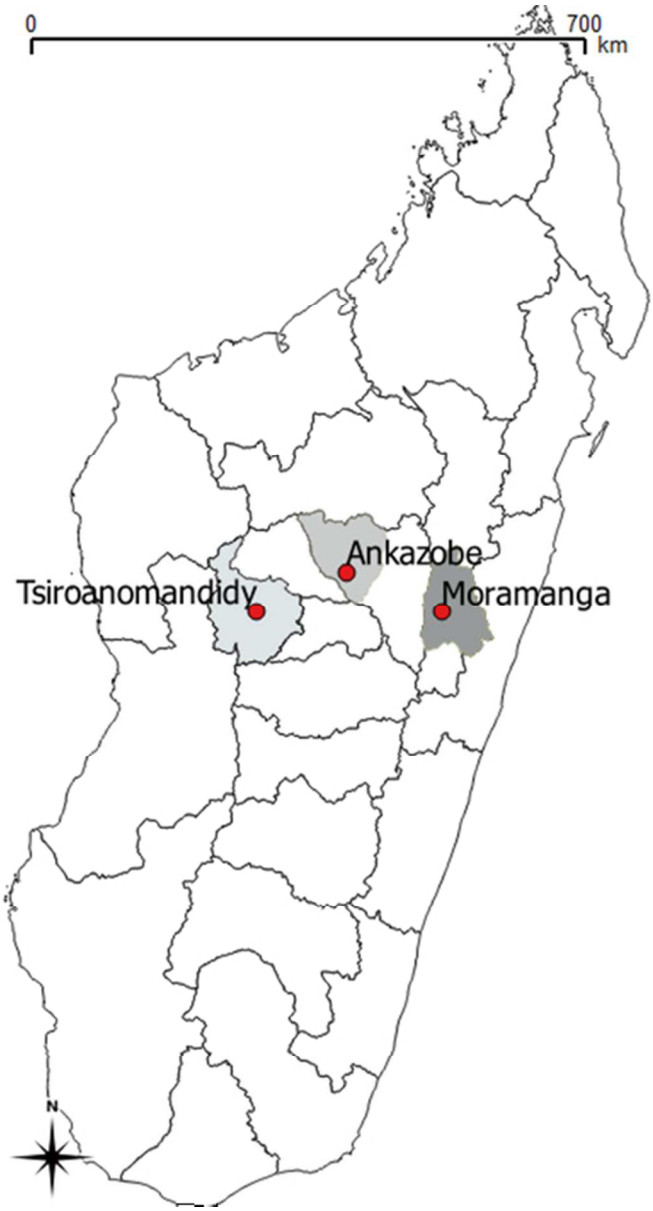

Fig. 1- The three study sites in Madagascar. Red dots design the study sites. Engorged and gravid females of Cx. antennatus and An. coustani were captured in three different sites in Madagascar: in Moramanga (18°51'27.60"S; 48° 7'40.20"E) located in the Alaotra-Mangoro region, in Ankazobe (18°24'16.81"S;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