JVI Accepts, published online ahead of print on 26 March 2014
J. Virol. doi:10.1128/JVI.00370-14

High vector competence of *Aedes aegypti* and *Aedes albopictus* from ten American countries as a crucial factor of the spread of Chikungunya

Anubis Vega-Rúa,[a,b] Karima Zouache,[a] Romain Girod,[c] Anna-Bella Failloux,[a,*] Ricardo Lourenço-de-Oliveira[a,d,*]

[a] Institut Pasteur, Department of Virology, Arboviruses and Insect Vectors, 25-28 rue du Docteur Roux, 75724 Paris, cedex 15, France.

[b] Université Pierre et Marie Curie, Cellule Pasteur UPMC, 25-28 rue du Dr Roux, 75724 Paris cedex 15, France.

[c] Institut Pasteur de la Guyane, Unité d'Entomologie médicale, Avenue Pasteur, BP 6010, 97306 Cayenne Cedex, Guyane française.

[d] Laboratório de Transmissores de Hematozoários, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil 4365, Rio de Janeiro, 21045-900, Brazil.

[*] Corresponding authors:

Ricardo Lourenço-de-Oliveira: lourenco@ioc.fiocruz.br, ricardo.lourenco-de-oliveira@pasteur.fr

Anna-Bella Failloux: anna-bella.failloux@pasteur.fr

Running title: Chikungunya in the Americas

Abstract: 247 words

Text: 5117 words

ABSTRACT

Chikungunya virus (CHIKV) causes a major public health problem. In 2004, CHIKV began an unprecedented global expansion and has been responsible for epidemics in Africa, Asia, islands in the Indian Ocean region, and surprisingly, in temperate regions such as Europe. Intriguingly, no local transmission of chikungunya virus (CHIKV) has been reported in the Americas until recently despite the presence of vectors and annually-reported imported cases. Here, we assessed the vector competence of 35 American *Ae. aegypti* and *Ae. albopictus* populations to three CHIKV genotypes. We also compared the number of viral particles of different CHIKV strains in mosquito saliva at two different times post-infection. Primarily, viral dissemination rates were high for all mosquito populations irrespective of the tested CHIKV isolate. In contrast, differences in transmission efficiency (TE) were underlined in populations of both species through the Americas suggesting the role of salivary glands in selecting CHIKV for highly efficient transmission. Nonetheless, both mosquito species were capable to transmit all three CHIKV genotypes, and TE reached alarming rates as high as 83.3% and 96.7% in *Ae. aegypti* and *Ae. albopictus* populations, respectively. *Ae. albopictus* better transmitted the epidemic mutant strain CHIKV_0621 of the East-Central-South African (ECSA) genotype than did *Ae. aegypti*, whereas this latter species was more capable of transmitting the original ECSA CHIKV_115 strain and also the Asian genotype CHIKV_NC. Therefore, a high risk of establishment and spread of CHIKV throughout the tropical, subtropical and even temperate regions of the Americas is more real than ever.

47 **IMPORTANCE**

48 Until recently, the Americas have never reported chikungunya (CHIK) autochthonous
49 transmission despite its global expansion beginning in 2004. Large regions of the continent
50 are highly infested with *Ae. aegypti* and *Ae. albopictus* and millions of dengue (DEN) cases
51 are annually recorded. Indeed, DEN and CHIK viruses share the same vectors. Due to a recent
52 CHIK outbreak affecting Caribbean islands, the need for a Pan-American evaluation of vector
53 competence was compelling as a key parameter in assessing the epidemic risk. We
54 demonstrated for the first time that *Ae. aegypti* and *Ae. albopictus* populations throughout the
55 continent are highly competent to transmit CHIK irrespective to the viral genotypes tested.
56 The risk of CHIK spreading throughout the tropical, subtropical and even temperate regions
57 of the Americas is more than ever a reality. In light of our results, local authorities should
58 immediately pursue and reinforce epidemiological and entomological surveillance to avoid a
59 severe epidemic.

60

61 **Key words:** Americas, *Aedes aegypti*, *Aedes albopictus*, chikungunya virus, emergence.

62

63

INTRODUCTION

Chikungunya virus (CHIKV) is an alphavirus in the family *Togaviridae* that is transmitted by mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus* within an urban cycle. Since 2004, CHIKV has reemerged in the Indian Ocean Islands and has caused severe epidemics in several countries in tropical and subtropical regions in Africa and Asia, as well as in temperate Mediterranean areas in Europe (1).

Aedes aegypti is widespread in the Americas, where it is the only confirmed natural dengue virus (DENV) vector (2). Although its geographical distribution is more limited, *Ae. albopictus* is considered a potential vector in the Americas due to the high vector competence of local populations to DENV (3,4). More than two millions dengue cases are annually reported in the American continent each year (5). The most critical epidemiological situation is that described for South America, which reported more than 1.5 million dengue cases in 2013, with an incidence rate of more than 650 cases/100,000 inhabitants in the South Cone alone (6). Such an epidemiological scenario points to the weakness of mosquito control activities and the high receptivity to introduction and spread of other arboviroses transmitted by both mosquito species like CHIKV in other parts of the continent (1,7,8). In fact, as CHIKV and DENV share the same mosquito vector species, epidemic waves caused by both viruses affect the same regions and human co-infections may occur (9,10). Moreover, the intensification of intercontinental travels with recurrent returns of dozens of viremic CHIKV cases from affected areas -that may bypass the surveillance systems due to the clinical similarities with other viruses circulating in the Americas- exemplifies the vulnerability of this continent to CHIKV epidemics (11,12). Indeed, Brazil, Canada, USA, French Guiana, and the French West Indies (Guadeloupe and Martinique) have reported several imported CHIKV cases since its re-emergence in 2004 (6,13).

Intriguingly, until December 2013, autochthonous CHIKV transmission has never been reported in the Americas, a continent where all the conditions are apparently suitable for its establishment: (i) it is a virgin continent for CHIKV, (ii) the main mosquito vectors of CHIKV, *Ae. aegypti* and *Ae. albopictus,* are present with high densities in most areas, (iii) imported cases are annually reported in periods of high mosquito density and activity, and (iv) temperature and environmental conditions of large tropical and subtropical zones are favorable to mosquito development and activity as well as to viral replication in the vector (11,14). In the early December 2013, two laboratory-confirmed autochthonous CHIKV cases were reported in the French territory of Saint-Martin Island in the Caribbean (6). Very rapidly, an epidemic was established on the island with almost 2030 clinical cases and more than 765 confirmed cases today, and subsequently, some CHIKV cases were detected in Martinique, Guadeloupe, Saint-Barthelemy, and also French Guyana (15). Therefore, CHIKV is progressively spreading putting at a high epidemic risk the vastly-infested *Ae. aegypti* and *Ae. albopictus* areas of the Americas.

To achieve efficient transmission, numerous factors regarding the invertebrate and the vertebrate hosts, the virus, and the environmental conditions must ideally converge (16). Concerning the mosquito host, vector competence is considered to be unique and characteristic for each virus-vector pair. Indeed differences of vector competence can be found between different populations belonging to a single insect vector species (17). Vector competence is a quantitative phenotypic parameter controlled by genetic characteristics of both vector and virus, which in turn is influenced by environmental conditions (18-20). Mosquito vector competence to CHIKV and DENV seems to be determined by genotype-by-genotype interactions, in which successfull transmission depends on some specific combination of mosquito and viral genetic characteristics (21-26). CHIKV has four major lineages: East-Central-South Africa (ECSA), West Africa, Asian, and the Indian Ocean, a

monophyletic lineage descendant from the ECSA group (27). The CHIKV lineages have displayed distinct transmission efficiencies in mosquito vector species and populations (25,28, 29). Throughout the 2005-2006 CHIKV epidemic in the Indian Ocean region, a CHIKV lineage strain harboring a substitution of an alanine to valine at position 226 of the E1 envelope glycoprotein (E1-A226V) was better transmitted by *Ae. albopictus* (22,25,30). It was later shown that other positions in the E2 glycoprotein exert epistatic effects on the position E1-226V (23,24) and some substitutions can block the adaptation of the E1-226V to *Ae. albopictus*. These epistatic interactions are lineage specific.

Determining vector competence of mosquito populations is a key parameter in evaluating the risk of CHIKV transmission and spread. Given the alarming epidemiological situation due to the very recent chikungunya outbreak affecting the Caribbean islands, the need for evaluating the vector competence of American mosquito populations is compelling. Until now, studies were only limited to mosquitoes from the USA and the French Caribbean (31-34). With the aim of understanding the factors that may influence CHIKV emergence in the Americas and the risk of CHIKV epidemic spreading throughout the continent, we carried out a comprehensive Pan-American evaluation of vector competence of 35 *Ae. aegypti* and *Ae. albopictus* populations from 10 countries towards three CHIKV isolates belonging to two distinct lineages.

MATERIALS AND METHODS

Ethics Statement. The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live animals [see permit numbers at http://webcampus.pasteur.fr/jcms/c_97619/agrements-des-animaleries] in appliance of the French and European regulations on care and protection of the Laboratory Animals. This

study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Institut Pasteur. No specific permits were required for the described field studies in locations which are not protected in any way and did not involve endangered or protected species.

Mosquitoes. Thirty five mosquito populations collected in 10 countries from North, Central and South Americas were used: 22 populations of *Ae. aegypti* and 13 of *Ae. albopictus* (Figure 1, Table 1). The mosquitoes were field-collected in 2012 with ovitraps (10-58 per collection site). The mosquito collection sites were strategically chosen in order to essentially represent the diverse climates, environments, ecotopes and dengue epidemiological history across the American continent. The field collected eggs were immersed in water for hatching; larvae were split by 100-150 individuals per pan and fed with yeast tablets. Emerging adults were maintained in cages at 28°±1°C with a 14h:10h light:dark cycle, 80% relative humidity, and supplied with a 10% sucrose solution. The F1 generation was used for all infection assays.

Viral Strains. Three CHIKV isolates belonging to two distinct lineages were used: two CHIKV isolates from La Réunion and one from New Caledonia. The isolates from La Réunion were the strains (i) CHIKV 05.115 (CHIKV_115) and (ii) CHIKV 06.21 (CHIKV_0621), both isolated in 2005 (35) and provided by the French National Reference Center for Arboviruses at the Institut Pasteur in Paris. The amino-acid consensus sequence of these strains differed only by a single substitution: CHIKV_115 has an alanine at position 226 of the E1 envelope glycoprotein (E1-226A), whereas CHIKV_0621 harbors a valine at the same position (E1-226V). It has been shown the E1-A226V substitution is located in a region known to be involved in viral entry via fusion with endosomal membranes (36). Both strains have an alanine at position 98 of the E1 glycoprotein (E1-98A) that has been shown to exert no negative epistatic effects on the position E1-226; the position E1-98 is located at the base

of the fusion loop and presumably modulates the kinetics of the pH-dependent conformational changes and fusion reaction in the endosomal compartment (37). Viral titer estimated by serial 10-fold dilutions on Vero cells was 10^9 plaque forming units (pfu)/mL for both CHIKV_115 and CHIKV_0621. Both strains were isolated on *Ae. albopictus* C6/36 cells from human serum or viral stocks and were produced following three passages on *Ae. albopictus* C6/36 cells then harvested and stored at -80°C until use for the mosquito experimental infection assays. The New Caledonia CHIKV strain referenced as NC/2011-568 (CHIKV_NC), was isolated in 2011 (28, 37) and provided by the Institut Pasteur of New Caledonia. Phylogenetic analysis using the complete CHIKV_NC genome nucleotide sequence demonstrated that CHIKV_NC belongs to the Asian lineage, displaying 98.1% nucleotide identity with other isolates of the Asian cluster of CHIKV phylogeny. CHIKV_NC strain has an alanine at position E1-226 (E1-226A) and a threonine at position E1-98 (E1-98T). It has been shown that in contrast with the ECSA genotype, the substitution E1-98T exerts a negative epistatic interaction leading to block the ability of Asian CHIKV strains to adapt to *Ae. albopictus* via the E1-A226V substitution (24). The whole genome sequence of CHIKV_NC is available on GenBank under accession no. HE806461. CHIKV_NC 2nd passage was used for the experimental infections of mosquitoes. The titer of CHIKV_NC stocks was $10^{8.1}$ pfu/mL.

Mosquito Oral Infections. Five to seven day-old females were fed on an infectious blood-meal containing 2 mL of washed rabbit erythrocytes, 1 mL of viral suspension supplemented with a phagostimulant (ATP) at a final concentration of 5 mM. The titer of all performed infectious blood-meals was $10^{7.5}$ pfu/mL. Mosquito feeding was limited to 50 min. After the infectious blood-meal, non-engorged females were discarded. Fully engorged females were transferred in cardboard containers and maintained with 10% sucrose at 28°±1°C. All 35

188 mosquito populations were challenged with the CHIKV_0621 strain (13 *Ae. albopictus* and
189 22 *Ae. aegypti* populations) whereas 22 populations (9 *Ae. albopictus* and 13 *Ae. aegypti*)
190 were challenged with the CHIKV_115 strain and 6 populations (3 *Ae. albopictus* and 3 *Ae.*
191 *aegypti*) with CHIKV_NC. Mosquito populations from the same location were
192 simultaneously tested with the CHIKV_0621 and CHIKV_115 strains.
193
194 **Dissemination and Transmission Analysis.** Batches of ~30 mosquitoes of each combination
195 of mosquito population-virus strain were analyzed at days 7 and 10 post-infection (pi) for all
196 the CHIKV strains tested. Days pi were defined according to the kinetics of CHIKV
197 dissemination and transmission efficiencies in *Ae. albopictus* from Paquetá, Rio de Janeiro,
198 Brazil (maximum at day 7 pi and slight decrease by day 10; see Figure 2). To estimate viral
199 dissemination, heads were removed from mosquitoes and ground in 250 µL of Leibovitz L15
200 medium (Invitrogen) supplemented with 2% Fetal Bovine Serum (FBS) for further
201 inoculation onto cell C6/36 *Ae. albopictus* cell culture in 96-well plates. After incubation at
202 28°C for 3 days, plates were stained using hyper-immune ascetic fluid specific to CHIKV as
203 primary antibody. Alexa Fluor® 488 goat anti-mouse IgG was used as the second antibody
204 (Life technologies ^{TM}).
205 To estimate viral transmission, saliva was collected from individual mosquitoes as described
206 in (38). For that, wings and legs were removed from each mosquito and the proboscis was
207 inserted into a 20 µL tip containing 5 µL of FBS. After 45 min of salivation, FBS containing
208 saliva was expelled into 45 µL of Leibovitz L15 medium for titration. One limitation of this
209 technique is that the volume of saliva delivered by females could not be estimated.
210 Dissemination efficiency corresponds to the proportion of mosquitoes with virus detected in
211 heads among tested ones (i.e., engorged mosquitoes which have survived until the day of
212 examination). Transmission efficiency corresponds to the proportion of mosquitoes with virus

in the saliva among tested ones (i.e., surviving females including females unable to disseminate the virus and those able to disseminate). The number of infectious particles per saliva was estimated by titration using focus fluorescent assay on C6/36 *Ae. albopictus* cells. Samples were serially diluted and inoculated onto C6/36 cells in 96-well plates, following incubation at 28°C for 3 days. Then, plates were stained as explained above.

Statistical Analysis. Statistical analyses were performed with STATISTICA 8 software (Statsoft Inc, USA). The numbers of infectious particles in saliva were compared using the Kruskal-Wallis test. Dissemination and transmission efficiencies were compared using Chi-square test. Kruskal-Wallis Z multiple comparison test was used to compare more than 5 dissemination and transmission efficiency rates.

RESULTS

Dissemination efficiency. To measure the ability of American *Ae. aegypti* and *Ae. albopictus* to allow CHIKV to overcome the midgut barrier, dissemination efficiency (DE) was assessed for each pairing mosquito population-virus strain at days 7 and 10 pi (Tables 2 and 3).

All *Ae. aegypti* and *Ae. albopictus* populations showed similar DE values at days 7 and 10 pi for the three CHIKV isolates (Chi-square test: $p>0.05$). For CHIKV_0621, DE at day 7 pi ranged from 60% to 100% for *Ae. albopictus* and from 93.3% to 100% for *Ae. aegypti*. For CHIKV_115, DE at day 7 varied from 66.7% to 96.9% for *Ae. albopictus* and from 96.6% to 100% for *Ae. aegypti,* while for CHIKV_NC, DE ranged from 90% to 96.7% for *Ae. albopictus* and from 96.9% to 100% for *Ae. aegypti. Ae. aegypti* tested populations displayed similar DE values around 100% for the three CHIKV isolates (Chi-square test: $p>0.05$). Likewise, DE obtained for *Ae. albopictus* were extensively high although rates were

238 significantly heterogeneous for CHIKV_0621 (Chi-square test: $p<0.05$) and CHIKV_115
239 (Chi-square test: $p<0.05$). Thus, when comparing DE values for a given virus between the two
240 mosquito species sampled in a same location, no significant difference was found except for
241 MXC in Mexico when infected with CHIKV_0621 (Chi-square test: $p<0.05$) and
242 CHIKV_115 (Chi-square test: $p<0.05$), and for VRB in United States when infected with
243 CHIKV_115 (Chi-square test: $p<0.05$). In these three last cases, *Ae. aegypti* exhibited a
244 higher DE than *Ae. albopictus* collected in the same site whatever the viral strain. In addition,
245 no difference was observed in DE values between the three *Ae. aegypti* and *Ae. albopictus*
246 populations challenged with the CHIKV_NC isolate (Chi-square test: $p>0.05$).
247
248 **Transmission efficiency.** In order to determine the ability of American *Ae. aegypti* and *Ae.*
249 *albopictus* to sustain CHIKV transmission, we assessed the transmission efficiency (TE) at
250 days 7 and 10 pi. only TE values at day 7 pi were presented in Figures 3 and 4 (see Table S1
251 for TE values at day 10 pi). The TE values obtained for *Ae. aegypti* and *Ae. albopictus* were
252 highly heterogeneous and lower than DE values.
253 When mosquitoes were exposed to CHIKV_0621, TE values ranged from 13.3% to 96.7 % at
254 day 7 pi and 6.7% - 85.2 % at day 10 pi. *Ae. albopictus* better transmitted CHIKV_0621 than
255 *Ae. aegypti* at day 7 pi (mean ± CI: 44.7 ± 7.8 for *Ae. aegypti* and 55.8 ± 12.3 for *Ae.*
256 *albopictus*) and at day 10 pi (mean ± CI: 33.1 ± 6.2 for *Ae. aegypti* and 55.5 ± 12.0 for *Ae.*
257 *albopictus*). Within a same mosquito species, TE values were significantly different (Chi-
258 square test: $p<0.05$) at days 7 and 10 pi. When considering each of the 10 populations where
259 the two species co-exist (VRB, MXC, PAN, MAN, PNM, JRB, PAQ, VAZ, BEL, SAN), *Ae.*
260 *albopictus* exhibited a higher TE than *Ae. aegypti* when infected with CHIKV_0621 except
261 for the VRB population from Florida, United States (Figures 3 and 4, Table S1).

When mosquitoes were infected with CHIKV_115, TE values were comprised between 11.1% and 82.1% at day 7 pi and 10% - 76.7% at day 10 pi. *Ae. aegypti* better transmitted CHIKV_115 than *Ae. albopictus* at day 7 pi (mean ± CI: 49.5 ± 10.3 for *Ae. aegypti* and 49.5 ± 13.6 for *Ae. albopictus*). Within a same mosquito species, TE values were significantly different (Chi-square test: $p<0.05$) at days 7 and 10 pi. When considering each of the four populations where the two species co-exist (VRB, MXC, PAN, and PAQ), one species did not present a clear-cut advantage on the other to transmit CHIKV_115 (Figures 3 and 4, Table S1).

Interestingly, among the eight *Ae. albopictus* populations simultaneously challenged with CHIKV_0621 and CHIKV_115, four showed unexpected lower TE for CHIKV_115 and one displayed equal rates (Figure 3, Table S1). Remarkably, TE rates were heterogeneous even between *Ae. albopictus* populations geographically close, i.e. from Rio de Janeiro, Brazil (JRB, PAQ, BEL, VAZ) when exposed to the same CHIKV_0621 isolate (Figures 3 and 4).

Lastly, when mosquitoes were exposed to the CHIKV_NC, TE values varied from 30% to 83.3% at day 7 pi, and 26.7%-53.3% at day 10 pi. *Ae. aegypti* better transmitted CHIKV_NC than *Ae. albopictus* at day 7 pi (mean ± CI: 64.5 ± 20.7 for *Ae. aegypti* and 48.9 ± 25.1 for *Ae. albopictus*). Within a same mosquito species, TE values were significantly different (Chi-square test: $p<0.05$) at day 7 and not at day 10 pi (Chi-square test: $p>0.05$) (see Table S1).

We also found that 23% - 56% mosquitoes collected in temperate regions, *Ae. albopictus* TYS (Tyson, United States), *Ae. aegypti* SAL (Salto, Uruguay) and BUE (Buenos Aires, Argentina) were able to efficiently transmit CHIKV_0621. Moreover, *Ae. aegypti* from the last two sites of the Southern Cone were also competent to efficiently transmit CHIKV_0115 and CHIKV_NC at day 7 pi, respectively (SAL = 70% for CHIKV_115; BUE = 48.3% for CHIKV_115 and 63.6% for CHIKV_NC).

Intensity of transmission. The intensity of viral transmission can be calculated by estimating the viral load in saliva collected from mosquitoes. When infected with CHIKV_0621 isolate, the number of viral particles in saliva ranged from 0.4 to 4.4 $\log_{10}$ particles for *Ae. albopictus* and from 0.4 to 5.1 $\log_{10}$ for *Ae. aegypti*. Concerning mosquitoes infected with CHIKV_115 isolate, the number of viral infectious particles varied from 0.4 to 4.7 $\log_{10}$ for *Ae. albopictus* and from 0.4 to 5.0 $\log_{10}$ for *Ae. aegypti.* For mosquitoes exposed to CHIKV_NC, the viral load in saliva ranged from 0.4 to 2.9 $\log_{10}$ for *Ae. albopictus* and from 0.4 to 4.2 $\log_{10}$ particles for *Ae. aegypti* (Figure 5). Viral loads of the three tested CHIKV strains were equivalent in *Ae. aegypti* populations, whereas *Ae. albopictus* displayed a slightly lower titer when challenged with CHIKV_NC in comparison to CHIKV_0621 and CHIKV_115, both at day 7 pi. Viral loads were highly heterogeneous between individuals belonging to the same population and infected with a given viral strain, but the mean calculated for each mosquito population was roughly similar overall. Indeed, when comparing viral load in saliva between mosquito strains for a given virus at day 7 and 10 pi (Figures 5 and S1), no significant differences were found either for *Ae. aegypti* or *Ae. albopictus* (Kruskal-Wallis test: $p>0.05$), except for *Ae. albopictus* challenged with CHIKV_115.

DISCUSSION

All 35 populations of *Ae. aegypti* and *Ae. albopictus* collected throughout the Americas were susceptible to CHIKV infection by all three tested genotypes. Thus, temperate as well as tropical and subtropical Northern, Central and Southern American *Aedes* mosquitoes are efficient CHIKV vectors. *Ae. albopictus* better transmitted the epidemic CHIKV_0621 strain isolated on La Réunion Island in 2006 (35) than *Ae. aegypti,* whereas this latter species was more capable to transmit the original strain CHIKV_115, both belonging to the ECSA

genotype (39). The Asian genotype represented by the CHIKV_NC strain (28) was better transmitted by *Ae. aegypti,* although it was also efficiently transmitted by *Ae. albopictus*.

Most American *Aedes* mosquitoes are highly susceptible to CHIKV

More than 60% of mosquitoes per population were able to disseminate CHIKV after crossing the midgut barrier (i.e., entry in epithelial cells, viral replication and release of virions from the midgut basal lamina). Thus after being ingested with a blood-meal provided at a titer of $10^{7.5}$ pfu/mL, CHIKV succeeded in disseminating within the mosquito hemocele which is an essential prerequisite for transmission. It has been shown that a titer of ~10^4 pfu/mL in monkeys was sufficient enough to infect mosquitoes (40). CHIKV transmission was highly heterogeneous in American mosquitoes, ranging from 11.1% to 96.7% at day 7 pi when considering all CHIKV strains. It should be underlined that we are not able to provide a control of salivation and we hypothesize that a CHIKV-negative saliva did not correspond to mosquitoes unable to salivate but to mosquitoes delivering a non-infected saliva. As expected from previous studies (22,25,41,30), *Ae. albopictus* better transmitted the epidemic strain CHIKV_0621 of the ECSA genotype than *Ae. aegypti*, even in cases where both mosquito species cohabit. *Ae. aegypti* transmitted preferentially CHIKV_115 and also, the Asian genotype CHIKV_NC in accordance with previous findings (28). CHIKV Asian strains have a particular E1-98T substitution which constrains CHIKV adaptation to *Ae. albopictus via* E1-A226V mutation (24). *Ae. aegypti* are more abundant in the Americas than *Ae. albopictus* mosquitoes and the E1-98T substitution of CHIKV viral strains does not have a negative effect on CHIKV interaction with *Ae. aegypti.* Thus, CHIKV Asian strains together with the CHIKV ECSA strains, represent a real danger to the Americas. Intriguingly, the CHIKV strain isolated during the last outbreak in the Caribbean also belongs to the Asian genotype (42) primarily transmitted in the past by *Ae. aegypti*. Although the intensity of transmission is

highly variable between mosquitoes, the mean number of viral particles delivered by mosquitoes was quite similar for each combination mosquito strain and viral strain.

Mosquitoes collected in tropical Latin America, Panama, Venezuela, Brazil, Bolivia, Paraguay, Argentina and Uruguay showed the highest transmission efficiency with up to 10,000 viral particles detected in mosquito saliva. Interestingly, mosquitoes from the main Brazilian city of Rio de Janeiro showed high transmission efficiencies. For example, 96.7% of *Ae. albopictus* JRB were able to transmit CHIKV_0621 (see Table S1). Moreover, the extrinsic incubation period of CHIKV, i.e. the time necessary for the virus to be detected in saliva ready for transmission after being ingested with the blood-meal (43), in both mosquito species is quite short (38). Indeed, an *Ae. albopictus* population from Rio de Janeiro (PAQ) was able to transmit infectious viral particles as rapidly as 2 days pi (Figure 2). Therefore, the risk of CHIKV establishment in densely populated cities such as Rio de Janeiro hosting more than 6 million people and infested by anthropophilic *Aedes* mosquitoes should be considered very high.

Mosquitoes from temperate Americas are potentially capable of sustaining CHIKV transmission

The ability of CHIKV to extend its natural range of distribution to include temperate regions was exemplified by the Italian outbreak in 2007 and the French local/autochthonous cases in 2010 (44,45). In the Americas, more than one hundred imported CHIKV cases were detected in the United States between 1995 and 2009 (11). Some of them developed a viremia high enough to infect mosquitoes. We found that 56.7% of *Ae. albopictus* TYS from Tyson (United States) and 83.3% of *Ae. aegypti* SAL from Salto (Uruguay) were able to transmit CHIKV_0621 at day 7 pi (see Table S1). Transmission efficiencies were lower for *Ae. aegypti* BUE from Buenos Aires (Argentina) (i.e.,23.3%, see Figure 3, Table S1) but higher when

infected with the CHIKV_NC Asian genotype (i.e., 63.6%, see Figure 3, Table S1). Therefore, the establishment of CHIKV in temperate American countries is not simply a fiction even if less than 30% of both mosquito species collected in the South of United States (VRB, Florida) were able to transmit CHIKV_0621. It has been found that *Ae. albopictus* from Florida are more competent vectors of CHIKV than *Ae. aegypti* (31-33). Outbreaks of DENV, also transmitted by *Aedes* mosquitoes, have occurred in Texas and Florida in the past years (46), reinforcing the risk of epidemics due to imported arboviruses in the United States. Local transmission of CHIKV could be maintained if the virus is introduced in the right place at the right time. Taken together, these findings underline the high variation of susceptibility to CHIKV of American mosquitoes, calling for including other factors (biological and environmental) in assessing transmission potential risk (47). Moreover, mosquito genetic structure should be promptly investigated. Phylogenetic analysis of both mosquito species should bring additional information on colonization history of *Ae. aegypti* and *Ae. albopictus* in the different countries of Americas (48,49). *Ae. aegypti* was most likely introduced in North America during the slave trade (50) while *Ae. albopictus* was established in 1985 in the United States (51) probably introduced in shipments of used tires from Japan (52), and in Brazil in 1986 (53) probably arriving from tropical Asia (52).

The fear becomes a reality

Still absent until very recently, CHIKV has been detected for the first time in the Americas in late December 2013. Currently, among the 2030 suspected CHIKV cases from the island of Saint-Martin in the Caribbean, more than 765 were confirmed positive to CHIKV by serology (15). The virus then spread to neighboring islands: Saint-Barthelemy with 380 cases, Martinique with 3940 cases, Guadeloupe with 1460 cases. Until now, 10 autochthonous cases have been reported in French Guiana which maintains a daily air link with the two other

French Overseas territories of Guadeloupe and Martinique. We previously showed that *Ae. aegypti* from French Guiana and French West Indies were highly competent to disseminate CHIKV and mosquito populations collected in dense housing environments exhibited the highest susceptibility (34). Thus, the risk of CHIKV spread and establishment is real and should concern all areas in the Americas where the vector mosquitoes are present.

Co-circulation of CHIKV and DENV could have great implication on human health

Interestingly, DENV is still circulating in the Caribbean together with CHIKV. Cases of co-infection DENV-CHIKV in patients have been first reported in 1967 (54) and since the emergence of CHIKV, reports of co-infections are increasing (10,55-63). Both viruses are transmitted by the same mosquito vectors, *Ae. aegypti* and *Ae. albopictus*. Co-infection of a mosquito vector by two viruses can occur after two successive infectious blood-meals taken on two different viremic hosts or after a single blood-meal taken on a co-infected host. It has been shown that CHIKV and DENV can be delivered together in one mosquito bite (64). As co-infections were a quite common phenomenon, consequences on the clinical presentation of the disease are expected.

Finally, the assessment of vector competence should be considered as a prerequisite to better evaluate the potential risk of CHIKV outbreaks once the virus is introduced from endemic regions. The numerous imported CHIKV viremic cases presaged the potential importance of this emerging arbovirus for the Americas where both mosquito species are well established. In light of epidemics now starting in the Caribbean, it remains imperative to pursue and reinforce epidemiological and entomological surveillance actions and control against the mosquitoes, *Ae. aegypti* and *Ae. albopictus*.

ACKNOWLEDGMENTS

We thank Anayansi C Valderrama, Carlos Eduardo Borda, Darío Vezzani, Daniel Sánchez-Guillén, Eduardo A. Lestani, Fátima Domingos, Gabriel Sylvestre, Gabriela Willat, Glenda Velásquez, Ima Braga, José Bento Lima, L. Phil Lounibos, Lorenzo Carceres, Marcelo Celestino dos Santos, Márcia Gonçalves de Castro, Maria de Lourdes Macoris, Mário Navarro, Nidia Martínez Acosta, Maria C Carrasquilla, Maria Ignez L. Bersot, Mirian G Palomino Salcedo, Mirko Rojas-Cortez, Oscar D. Salomon, Rafael Maciel-de-Freitas, Romeo Humberto Montoya, Sérgio L. Bessa Luz, Steve Juliano, Tamara Chávez Espada, Teresa F. Silva-do-Nascimento, Yasmin Rubio-Palis for their help in mosquito sampling in the field and/or mosquito rearing; Marie Vazeille and Laurence Mousson for technical advices; Myrielle Dupont-Pouzeyrol for providing CHIKV_NC, Peter Sahlins and Henri Jupille for correcting the manuscript.

This work was funded by CNPq (grants 202106/2011-0 and 306340/2009-7), Instituto Oswaldo Cruz, Institut Pasteur (ACIP grant A-03-2012) and the French Government's Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID). AVR was supported by the French Ministry of Superior Education and Research, KZ by the European Community's Seventh Framework Programme (FP7/2007 - 2 013) under the project "VECTORIE", EC grant agreement number 261466 and the Foundation Inkermann (Fondation de France).

Competing interests

The authors declare that they have neither competing interests nor conflict of interest related to this article.

Authors' contribution

RLO and ABF conceived the study. RLO, AVR and KZ carried out experimental infections of mosquitos and performed titration assays. AVR, RLO and ABF drafted the manuscript. KZ and RG helped to draft and to revise the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

1. **Staples JE, Breiman RF, Powers AM.** 2009. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. Clin. Infect. Dis. **49:**942-948.
2. **Lourenço-de-Oliveira R, Vazeille M, Filipis AMB, Failloux A-B.** 2004. *Aedes aegypti* in Brazil: genetically differentiated populations with high susceptibility to dengue and yellow viruses. Trans. R. Soc. Trop. Med Hyg. **98:**43-54.
3. **Lourenço-de-Oliveira R, Vazeille M, de Filippis AM, Failloux AB.** 2003. Large genetic differentiation and low variation in vector competence for dengue and yellow fever viruses of *Aedes albopictus* from Brazil, the United States, and the Cayman Islands. Am. J. Trop. Med. Hyg. **69:**105-114.
4. **Benedict MQ, Levine RS, Hawley WA, Lounibos LP.** 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne Zoonotic Dis. **7:**76-85.
5. Organización Panamericana de la Salud. Dengue. [cited 6 Jan 2014]. http://new.paho.org/hq/index.php?option=com_content&view=article&id=264&Itemid=363&lang=es.
6. Organización Panamericana de la Salud. Epidemiological alert. Chikungunya fever, 9 December 2013. [cited 6 Jan 2014].

(http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&gid=23806+&Itemid=999999&lang=en).

7. **Figueiredo LT.** 2007. Emergent arbovirus in Brazil. Rev. Soc. Bras. Med. Trop. **40:**224-229.
8. **Maciel-de-Freitas R, Aguiar R, Bruno RV, Guimarães MC, Lourenço-de-Oliveira R, Sorgine MH, Struchiner CJ, Valle D, O'Neill SL, Moreira LA.** 2012. Why do we need alternative tools to control mosquito-borne diseases in Latin America? Mem. Inst. Oswaldo Cruz **107:**828-829.
9. **Mackenzie JS, Chua KB, Daniels PW, Eaton BT, Field HE, Hall RA, Halpin K, Johansen CA, Kirkland PD, Lam SK, McMinn P, Nisbet DJ, Paru R, Pyke AT, Ritchie SA, Siba P, Smith DW, Smith GA, Van den Hurk AF, Wang LF, Williams DT.** 2012. Emerging viral diseases of Southeast Asia and the Western Pacific. Emerg. Infect. Dis. **7:**497–504.
10. **Chahar HS, Bharaj P, Dar L, Guleria R, Kabra SK, Broor S.** 2009. Co-infections with chikungunya virus and dengue virus in Delhi, India. Emerg. Infect. Dis. **15:**1077-1080.
11. **Gibney KB, Fischer M, Prince HE, Kramer LD, St George K, Kosoy OL, Laven JJ, Staples JE.** 2011. Chikungunya fever in the United States: a fifteen year review of cases. Clin. Infect. Dis. **52:**e121-126.
12. **Chaves TS, Pellini AC, Mascheretti M, Jahnel MT, Ribeiro AF, Rodrigues SG, Vasconcelos PF, Boulos M.** 2012. Travelers as sentinels for chikungunya fever, Brazil. Emerg. Infect. Dis. **18:**529-530.
13. Center for Diseases Control. 2006. Chikungunya fever diagnosed among international travelers-United States, 2005-2006. Morb. Mortal. Wkly. Rep. 55:1040-1042.

14. **Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers F, George DB, Jaenisch T, Wint GRW, Simmons CP, Scott TW, Farrar JJ, Hay SI.** 2013. The global distribution and burden of dengue. Nature **496:**504-507.

15. CIRE Antilles Guyane. Le chikungunya dans les Antilles_Guyane. Le point épidémiologique, n. 8, 17-23 February 2014. [cited 2014 Feb 27]. http://www.invs.sante.fr/fr/Publications-et-outils/Points-epidemiologiques/Tous-les-numeros/Antilles-Guyane/2014/Situation-epidemiologique-du-chikungunya-dans-les-Antilles.-Point-au-27-fevrier-2014.

16. **Lambrechts L, Failloux AB.** 2012. Vector biology prospects in dengue research. Mem. Inst. Oswaldo Cruz **107:**1080-1082.

17. **Bennett KE, Olson KE, Muñoz ML, Fernández-Salas I, Farfán JA, Higgs S, Black WC, Beaty BJ.** 2002. Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States. Am. J. Trop. Med. Hyg. **67:**85-92.

18. **Failloux AB, Vazeille M, Rodhain F.** 2002. Geographic genetic variation in populations of the dengue virus vector *Aedes aegypti*. J. Mol. Evol. **5:**653-63.

19. **Black WC IV, Bennett KE, Gorrochótegui-Escalante N, Barrilas-Mury CV, Fernandez-Salas I, Munoz ML, Farfan-Ale JÁ, Olson KE, Beaty BJ.** 2002. Flavivirus susceptibility in *Aedes aegypti*. Arch. Med. Res. **33:**379–388.

20. **Tabachnick WJ.** 2013. Nature, nurture and evolution of intra-species variation in mosquito rrbovirus transmission competence. Int. J. Environ. Res. Public. Health. **10:**249-277.

21. **Bosio CF, Fulton RE, Salasek ML, Beaty BJ, Black WC IV.** 2000. Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti*. Genetics **156:**687–698.

22. **Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S.** 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. **3:**e201.

23. **Tsetsarkin KA, McGee CE, Volk SM, Vanlandingham DL, Weaver SC, Higgs S.** 2009. Epistatic roles of E2 glycoprotein mutations in adaption of chikungunya virus to *Aedes albopictus* and *Ae. aegypti* mosquitoes. PLoS ONE **4:**e6835.

24. **Vazeille M, Moutailler S, Coudrier D, Rousseaux C, Khun H, Huerre M, Thiria J, Dehecq JS, Fontenille D, Schuffenecker I, Despres P, Failloux AB.** 2007. Two chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. PLoS ONE **2:**e1168.

25. **Lambrechts L.** 2011. Quantitative genetics of *Aedes aegypti* vector competence for dengue viruses: towards a new paradigm? Trends Parasitol. **27:**111-114.

26. **Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, Nasar F, Schuh AJ, Holmes EC, Higgs S, Maharaj PD, Brault AC, Weaver SC.** 2010. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. J. Virol. **84:**6497-6504.

27. **Dupont-Rouzeyrol M, Caro V, Guillaumot L, Vazeille M, D'Ortenzio E, Thiberge JM, Baroux N, Gourinat AC, Grandadam M, Failloux AB.** 2012. Chikungunya virus and the mosquito vector *Aedes aegypti* in New Caledonia (South Pacific Region). Vector Borne Zoonotic Dis. **12:**1036-1041.

28. **Vega-Rua A, Zouache K, Caro V, Diancourt L, Delaunay P, Grandadam M, Failloux AB.** 2013. High efficiency of temperate *Aedes albopictus* to transmit chikungunya and dengue viruses in the Southeast of France. PLoS ONE **8:**e59716.

29. **Arias-Goeta C, Mousson L, Rougeon F, Failloux AB.** 2013. Dissemination and transmission of the E1-226V variant of chikungunya virus in *Aedes albopictus* are controlled at the midgut barrier level. PLoS ONE **8:**e57548.

30. **Reiskind MH, Pesko K, Westbrook CJ, Mores CN.** 2008. Susceptibility of Florida mosquitoes to infection with chikungunya virus. Am. J. Trop. Med. Hyg. **78:**422-425.

31. **Richards SL, Anderson SL, Smartt CT.** 2010. Vector competence of Florida mosquitoes for chikungunya virus. J. Vector Ecol. **35:**439-443.

32. **Pesko K, Westbrook CJ, Mores CN, Lounibos LP, Reiskind MH.** 2009. Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus. J. Med. Entomol. **46:**395-399.

33. **Girod R, Gaborit P, Marrama L, Etienne M, Ramdini C, Rakotoarivony I, Dollin C, Carinci R, Issaly J, Dusfour I, Gustave J, Yp-Tcha MM, Yébakima A, Failloux AB, Vazeille M.** 2011. High susceptibility to Chikungunya virus of *Aedes aegypti* from the French West Indies and French Guiana. Trop. Med. Int. Health. **16:**134-139.

34. **Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, Lavenir R, Pardigon N, Reynes JM, Pettinelli F, Biscornet L, Diancourt L, Michel S, Duquerroy S, Guigon G, Frenkiel MP, Bréhin AC, Cubito N, Desprès P, Kunst F, Rey FA, Zeller H, Brisse S.** 2006. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. **3:**e263.

35. **Voss JE, Vaney MC, Duquerroy S, Vonrhein C, Girard-Blanc C, Crublet E, Thompson A, Bricogne G, Rey FA.** 2010. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. Nature **468:**709-712.

36. **Tsetsarkin KA, Chen R, Leal G, Forrester N, Higgs S, Huang J, Weaver SC.** 2011. Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes. Proc. Natl. Acad. Sci. U. S. A. **108:**7872-7877.

37. **Alibert A, Pfannstiel A, Grangeon JP.** Chikungunya outbreak in New Caledonia in 2011. Status report as at 22 August 2011. Informa'Action n. 34. [cited 2014 Jan 7]. https://www.spc.int/phs/ENGLISH/Publications/InformACTION/IA34/Status_report_Chikungunya_Outbreak_New_Caledonia-22Aug2011.pdf.

38. **Dubrulle M, Mousson L, Moutailler S, Vazeille M, Failloux AB.** 2009. Chikungunya virus and *Aedes* mosquitoes: saliva is infectious as soon as two days after oral infection. PLoS ONE **4:**e5895.

39. **Powers AM, Logue CH.** 2007. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J. Gen. Virol. **88:**2363-2377.

40. **Turell MJ, Beaman JR, Tammariello RF.** 1992. Susceptibility of selected strains of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) to chikungunya virus. J. Med. Entomol. **29:**49-53.

41. **Martin E, Moutailler S, Madec Y, Failloux AB.** 2010. Differential responses of the mosquito *Aedes albopictus* from the Indian Ocean region to two chikungunya isolates. BMC Ecol. **10:**8.

42. **Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X.** 2014. Chikungunya in the Americas. Lancet **383:**514.

43. **Kramer LD, Ebel GD.** 2003. Dynamics of flavivirus infection in mosquitoes. Adv. Virus Res. **60:**187-232.

44. **Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, Panning M, Cordioli P, Fortuna C, Boros S, Magurano F, Silvi G, Angelini P, Dottori M, Ciufolini MG,**

Majori GC, Cassone A; CHIKV study group. 2007. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet **370:**1840-1846.

45. **Grandadam M, Caro V, Plumet S, Thiberge JM, Souares Y, Failloux AB, Tolou HJ, Budelot M, Cosserat D, Leparc-Goffart, Desprès P.** 2011. Chikungunya virus, southeastern France. Emerg. Infect. Dis. **17:**910-913.

46. Center for Diseases Control. Locally acquired dengue – Key West, Florida, 2009-2010. MMWR Morb Mort Wkly Rep. 2010; 59:577-581.

47. **Fontenille D, Failloux A-B, Romi R.** 2007. Should we expect Chikungunya and Dengue in Southern Europe? In: Emerging Pests and Vector-Borne Diseases in Europe (W Takken and BGJ Knols, Eds.). Wageningen Academic Publishers, Wageningen, The Netherlands, p.169-184.

48. **Bracco JE, Capurro ML, Lourenço-de-Oliveira R, Sallum MA.** 2007. Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence of multiple introductions. Mem. Inst. Oswaldo Cruz **102:**573-580.

49. **Mousson L, Dauga C, Garrigues T, Schaffner F, Vazeille M, Failloux AB.** 2005. Phylogeography of *Aedes (Stegomyia) aegypti* (L.) and *Aedes (Stegomyia) albopictus* (Skuse) (Diptera: Culicidae) based on mitochondrial DNA variations. Genet. Res. **86:**1-11.

50. **Powell JR, Tabachnick WJ.** 2013. History of domestication and spread of *Aedes aegypti* – A rewiew. Mem. Inst. Oswaldo Cruz **108** (Suppl I):11-17.

51. **Sprenger D, Wuithiranyagool T.** 1986. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J. Am. Mosq. Control Assoc. **2:**217–219.

52. **Hawley WA, Reiter P, Copeland RS, Pumpuni CB, Craig GB Jr.** 1987. *Aedes albopictus* in North America: probable introduction in used tires from northern Asia. Science **236:** 1114−1116.

606 53. **Consoli RAGB, Lourenço-de-Oliveira R.** 1994. Principais Mosquitos de
607 Importância Sanitária no Brasil. Rio de Janeiro: Fiocruz.
608 54. **Myers RM, Carey DE.** 1967. Concurrent isolation from patient of two arboviruses,
609 chikungunya and dengue type 2. Science **157:**1307–1308.
610 55. **Nayar SK, Noridah O, Paranthaman V, Ranjit K, Norizah K, Chem YK, Mustafa**
611 **B, Chua KB.** 2007. Coinfection of dengue virus and chikungunya virus in two
612 patients with acute febrile illness. Med. J. Malaysia **62:**335-336.
613 56. **Hapuarachchi HAC, Bandara KBAT, Hapugoda MD, Williams S,**
614 **Abeyewickreme W.** 2008. Laboratory confirmation of dengue and chikungunya co-
615 infection. Ceylon Med. J. **53:**104–105.
616 57. **Ratsitorahina M, Harisoa J, Ratovonjato J, Biacabe S, Reynes JM, Zeller H,**
617 **Raoelina Y, Talarmin A, Richard V, Louis Soares J.** 2008. Outbreak of dengue and
618 chikungunya fevers, Toasmasina, Madagascar, 2006. Emerg. Infect. Dis. **14:**1135-
619 1137.
620 58. **Schilling S, Emmerich P, Gu¨nter S, Schmidt-Chanasit J.** 2009. Dengue and
621 chikungunya virus co-infection in a German traveler. J. Clin. Virol. **45:**163–164.
622 59. **Leroy EM, Nkoghe D, Olomo B, Nze-Nkogue C, Becquart P, Grard G, Pourrut X,**
623 **Charrel R, Moureau G, Ndjoyi-Mbiguino A, De-Lamballerie X.** 2009. Concurrent
624 chikungunya and dengue virus infections during simultaneous outbreaks, Gabon,
625 2007. Emerg. Infect. Dis. **15:**591-593.
626 60. **Chang SF, Su CL, Shu PY, Yang CF, Liao TL, Cheng CH, Hu HC, Huang JH.**
627 2010. Concurrent isolation of chikungunya virus and dengue virus from a patient with
628 coinfection resulting from a trip to Singapore. J. Clin. Microbiol. **48:**4586-4589.
629 61. **Kalawat U, Sharma KK, Reddy SG.** 2011. Prevalence of dengue and chickungunya
630 fever and their co-infection. Indian J. Pathol. Microbiol. **54:**844-846.

631 62. **Kumar KJ, Manjunath VG, Shailashree M, Girish GN.** 2012. Coinfection with
632 dengue and chikungunya - a case report. J. Indian. Med. Assoc. **110:**749-752.
633 63. **Caron M, Paupy C, Grard G, Becquart P, Mombo I, Nso BB, Kassa Kassa F,**
634 **Nkoghe D, Leroy EM.** 2012. Recent introduction and rapid dissemination of
635 Chikungunya virus and Dengue virus serotype 2 associated with human and mosquito
636 coinfections in Gabon, central Africa. Clin. Infect. Dis. **55:**e45-53.
637 64. **Vazeille M, Mousson L, Martin E, Failloux AB.** 2010. Orally co-*Infected Aedes*
638 *albopictus* from La Reunion Island, Indian Ocean, can deliver both dengue and
639 chikungunya infectious viral particles in their saliva. PLoS Negl. Trop. Dis. **4:**e706.
640

Figure Legends

FIG 1 Mosquito populations tested. Color-code indicates localities where only *Ae. aegypti* (red), only *Ae. albopictus* (blue) and both *Ae. aegypti* and *Ae. albopictus* were collected (green). **TYS** Tyson (United States), **VRB** Vero Beach (United States), **MXC** Chiapas (Mexico), **PAN** Panamá (Panama), **DEL** Delta Amacuro (Venezuela), **TUM** Tumbes (Peru), **PUM** Punchana (Peru), **MAN** Manaus (Brazil), **STR** Santarém (Brazil), **PNM** Parnamirim (Brazil), **CAB** Campos Belos (Brazil), **CPG** Campo Grande (Brazil), **JRB** Jurujuba (Brazil), **PAQ** Paquetá (Brazil), **VAZ** Vaz Lobo (Brazil), **BEL** Belford Roxo (Brazil), **SAN** Santos (Brazil), **BMA** Monteagudo (Bolivia), **SDG** Salto del Guairá (Paraguay), **ASU** Asuncion (Paraguay), **SAL** Salto (Uruguay), **MIA** Misiones (Argentina), **ACO** Corrientes (Argentina), **BUE** Buenos Aires (Argentina).

FIG 2 Dissemination (A) and transmission efficiencies (B) of two CHIKV isolates and two clones of the respective viral isolates in *Ae. albopictus* from Paquetá, Rio de Janeiro (Brazil). At days 1, 2, 3, 7 and 10 after an infectious blood meal, mosquitoes were sacrificed and heads and saliva were collected for determination of their infectious status. Mosquito heads were individually ground in 250 µL Leibovitz L15 medium supplemented with 4%, following inoculation onto C6/36 *Ae. albopictus* cell monolayer in 96-well plates and incubation at 28°C for 3 days. Plates were fixed with 3.6% formaldehyde, washed three times with PBS and analyzed by indirect immunofluorescence assay (IFA). For saliva collection, each mosquito had wings and legs removed and the proboscis inserted into a 20 µL tip containing 5 µL of FBS. After 45 min of salivation, FBS containing saliva was expelled into 45 µL of Leibovitz L15 medium and inoculated onto C6/36 *Ae. albopictus* cell monolayer in 96-well plates. Plates were incubated and stained (IFA) as described in Materials and Methods.

Dissemination efficiency corresponds to the proportion of mosquito females with disseminated virus in head among the tested ones. Transmission efficiency corresponds to the proportion of mosquitoes with infectious saliva among the tested ones. CHIKV_0621: strain isolated from La Réunion (E1-226V substitution); CHIKV_115: strain isolated from La Réunion (E1-226A); CHIKV_0621 (V): clone corresponding to a single virus isolated from CHIKV_0621; CHIKV_115 (A): clone corresponding to a single virus isolated from CHIKV_115. Clones were provided by Arias-Goeta C.

FIG 3 Transmission efficiency of three CHIKV isolates in 35 *Ae. albopictus* and *Ae. aegypti* populations from 10 American countries at day 7 post-infection. After an infectious blood-meal, mosquitoes were sacrificed and saliva was collected from individual mosquitoes and titrated by focus fluorescent assay on C6/36 *Ae. albopictus* cells to determine infectious status. Transmission efficiency corresponds to the proportion of mosquitoes with infectious saliva among tested ones. Viral strains: CHIKV_0621 isolated from La Réunion (ECSA genotype, E1-226V and E1-98A substitution), CHIKV_115 isolated from La Réunion (ECSA genotype, E1-226A and E1-98A substitution) and CHIKV_NC isolated from New Caledonia (Asian genotype, E1-226A and E1-98T substitution). Mosquito populations (from North to South): **TYS** Tyson (United States), **VRB** Vero Beach (United States), **MXC** Chiapas (Mexico), **PAN** Panamá (Panama), **DEL** Delta Amacuro (Venezuela), **TUM** Tumbes (Peru), **PUM** Punchana (Peru), **MAN** Manaus (Brazil), **STR** Santarém (Brazil), **PNM** Parnamirim (Brazil), **CAB** Campos Belos (Brazil), **CPG** Campo Grande (Brazil), **JRB** Jurujuba (Brazil), **PAQ** Paquetá (Brazil), **VAZ** Vaz Lobo (Brazil), **BEL** Belford Roxo (Brazil), **SAN** Santos (Brazil), **BMA** Monteagudo (Bolivia), **SDG** Salto del Guairá (Paraguay), **ASU** Asuncion (Paraguay), **SAL** Salto (Uruguay), **MIA** Misiones (Argentina), **ACO** Corrientes (Argentina), **BUE** Buenos Aires (Argentina). Error bars show the confidence intervals (95%).

691

692 **FIG 4** Transmission efficiency of CHIKV_0621 and CHIKV_115 isolates in 35 *Ae. aegypti*
693 and *Ae. albopictus* populations from 10 American countries at day 7 post-infection.
694 Transmission efficiency corresponds to the proportion of mosquitoes with infectious saliva
695 among tested ones. Color-code indicates different degrees of transmission efficiency (TE):
696 yellow, mosquito strains with TE ≤ 30% (low TE); pale-orange, strains with 30% < TE < 70%
697 (moderated TE); red, strains with TE ≥ 70% (high TE). Viral strains: CHIKV_0621 isolated
698 from La Réunion (ECSA genotype, E1-226V substitution) and CHIKV_115 isolated from La
699 Réunion (ECSA genotype, E1-226A substitution. Mosquito populations (from North to
700 South): **TYS** Tyson (United States), **VRB** Vero Beach (United States), **MXC** Chiapas
701 (Mexico), **PAN** Panamá (Panama), **DEL** Delta Amacuro (Venezuela), **TUM** Tumbes (Peru),
702 **PUM** Punchana (Peru), **MAN** Manaus (Brazil), **STR** Santarém (Brazil), **PNM** Parnamirim
703 (Brazil), **CAB** Campos Belos (Brazil), **CPG** Campo Grande (Brazil), **JRB** Jurujuba (Brazil),
704 **PAQ** Paquetá (Brazil), **VAZ** Vaz Lobo (Brazil), **BEL** Belford Roxo (Brazil), **SAN** Santos
705 (Brazil), **BMA** Monteagudo (Bolivia), **SDG** Salto del Guairá (Paraguay), **ASU** Asuncion
706 (Paraguay), **SAL** Salto (Uruguay), **MIA** Misiones (Argentina), **ACO** Corrientes (Argentina),
707 **BUE** Buenos Aires (Argentina).

708

709 **FIG 5** Viral loads of three CHIKV isolates in saliva of *Ae. albopictus* and *Ae. aegypti*
710 mosquitoes from 35 populations from the Americas at day 7 post-infection. At day 7 after an
711 infectious blood-meal, mosquitoes were sacrificed and saliva was collected from individual
712 mosquitoes and titrated by focus fluorescent assay on C6/36 *Ae. albopictus* cells. Viral
713 strains: CHIKV_0621 isolated from La Réunion (ECSA genotype, E1-226V and E1-98A
714 substitution), CHIKV_115 isolated from La Réunion (ECSA genotype, E1-226A and E1-98A
715 substitution) and CHIKV_NC isolated from New Caledonia (Asian genotype, E1-226A and

716 E1-98T substitution). Mosquito populations (from North to South): **TYS** Tyson (United
717 States), **VRB** Vero Beach (United States), **MXC** Chiapas (Mexico), **PAN** Panamá (Panama),
718 **DEL** Delta Amacuro (Venezuela), **TUM** Tumbes (Peru), **PUM** Punchana (Peru), **MAN**
719 Manaus (Brazil), **STR** Santarém (Brazil), **PNM** Parnamirim (Brazil), **CAB** Campos Belos
720 (Brazil), **CPG** Campo Grande (Brazil), **JRB** Jurujuba (Brazil), **PAQ** Paquetá (Brazil), **VAZ**
721 Vaz Lobo (Brazil), **BEL** Belford Roxo (Brazil), **SAN** Santos (Brazil), **BMA** Monteagudo
722 (Bolivia), **SDG** Salto del Guairá (Paraguay), **ASU** Asuncion (Paraguay), **SAL** Salto
723 (Uruguay), **MIA** Misiones (Argentina), **ACO** Corrientes (Argentina), **BUE** Buenos Aires
724 (Argentina). Error bars refer to the standard error of mean titer for each pairing mosquito
725 population-virus strain.
726

727 **TABLE 1** Mosquito populations used. Populations are listed according to their country of collection,
728 from North to South.
729

Mosquito population	Collection site	Country	Coordinates	Mosquito species used	Climate	Dominant vegetation	Environment	History of Dengue incidence
TYS	Tyson Missouri	United States	38°31'N 90°33'W	AL	Temperate	Temperate grassland	Suburban	F
VRB	Vero Beach Florida	United States	27°35'N 80°22'W	AE/AL	Humid subtropical	Subtropical evergreen forest	Suburban	F
MXC	Tapachula	Mexico	14°53'N 92°15'W	AE/AL	Tropical wet and dry	Tropical deciduous forest	Suburban	M
PAN	Panamá/Colon	Panama	08°59'N 79°30W/ 09°21'N 79°53'W	AE/AL	Tropical wet and dry	Savana	Urban/ Suburban	L
DEL	Delta Amacuro Tucupita	Venezuela	09°03'N 62°02'W	AE	Tropical wet and dry	Savana	Suburban	L
PUM	Punchana Iquitos	Peru	03°43'S 73°15'W	AE	Tropical wet and dry	Amazon forest	Urban	H
TUM	Tumbes Huaquillas	Peru	03°29'S 80°15'W	AE	Arid	Desert	Suburban	L
MAN	Manaus	Brazil	03°06'S 60°03'W	AE/AL	Tropical wet	Amazon forest	Suburban	H
STR	Santarém	Brazil	02°25'S 54°42'W	AE/AL	Tropical wet	Amazon forest	Suburban	M
PNM	Parnamirim	Brazil	05°54'S 35°16'W	AE/AL	Semiarid	Transitional Tropical rainforest	Suburban	H
CAB	Campos Belos	Brazil	13°02'S 46°46'W	AE	Tropical wet and dry	Savana	Urban	L
BEL	Belford Roxo Rio de Janeiro	Brazil	22°45'S 43°24'W	AE/AL	Tropical wet and dry	Atlantic rain forest	Suburban	H
VAZ	Vaz Lobo Rio de Janeiro	Brazil	22°51'S 43°19W	AE/AL	Tropical wet and dry	Atlantic rain forest	Urban	H
JRB	Jurujuba Rio de Janeiro	Brazil	22°55'S 43°07'W	AE/AL	Tropical wet and dry	Atlantic rain forest	Suburban	L
PAQ	Paquetá Rio de Janeiro	Brazil	22° 45'S 43°06'W	AE/AL	Tropical wet and dry	Atlantic rain forest	Suburban island	M
SAN	Santos	Brazil	23°57'S 46°20'W	AE/AL	Tropical wet and dry	Atlantic rain forest	Suburban	M
CPG	Campo Grande	Brazil	20°27'S 54°37'W	AE	Tropical wet and dry	Savana	Urban	H
BMA	Monteagudo	Bolivia	19°48'S 63°57'W	AE	Tropical wet and dry	Mountain Forest	Urban	L
ASU	Asunción	Paraguay	25°18'S 57°37'W	AE	Tropical wet and dry	Chaco	Urban	M
SDG	Salto del Guairá	Paraguay	24°03'S 54°18'W	AE	Humid subtropical	Savana	Suburban	L
MIA	Misiones	Argentina	25°36'S 54°34'W	AL	Humid subtropical	Paranaense forest	Rural	L
ACO	Corrientes	Argentina	27°28'S 58°50'W	AE	Humid subtropical	Humid Chaco	Urban	M
BUE	Buenos Aires	Argentina	34°35'S 58°22W	AE	Temperate	Pampas	Urban	L
SAL	Salto	Uruguay	31°23'S 57°58'W	AE	Temperate	Pampa	Urban	F

730 AE: *Ae. aegypti;* AL: *Ae. albopictus;* F: Free; L: Low; M: Mediun; H: High
731

Table 2. Dissemination efficiency of three CHIKV isolates in 22 *Ae. aegypti* and 13 *Ae. albopictus* populations from 10 American countries at day 7 post-infection.

Country	Mosquito population	CHIKV_0621		CHIKV_115		CHIKV_NC	
		AE	AL	AE	AL	AE	AL
United States	TYS	ND	96.7% (30)	ND	83.3%(30)	ND	ND
	VRB	100% (30)	93.3% (30)	100% (18)	73.3% (30)*	ND	ND
Mexico	MXC	96.7% (30)	73.3% (30)*	96.7% (30)	66.7% (30)*	ND	ND
Panama	PAN	96.7% (30)	96.7% (30)	96.7% (30)	93.3% (30)	100% (30)	96.7% (30)
Venezuela	DEL	100% (23)	ND	100% (28)	ND	ND	ND
Peru	TUM	100% (30)	ND	ND	ND	ND	ND
	PUM	100% (30)	ND	100% (29)	ND	ND	ND
Brazil	MAN	100% (30)	96.7% (30)	ND	90.3% (31)	100% (30)	90% (30)
	STR	100%(30)	100% (30)	ND	88.4% (26)	ND	ND
	PNM	100% (30)	93.3% (30)	ND	ND	ND	ND
	CAB	100% (30)	ND	ND	ND	ND	ND
	CPG	100% (30)	ND	100% (30)	ND	ND	ND
	JRB	100% (30)	100% (30)	100% (30)	ND	ND	ND
	PAQ	100% (30)	87.1% (31)	100% (30)	96.9% (29)	ND	ND
	VAZ	100% (30)	91.3% (23)	ND	ND	ND	ND
	BEL	100% (30)	90.9%(22)	ND	ND	ND	ND
	SAN	93.3% (30)	100% (30)	ND	87.5% (8)	ND	ND
Bolivia	BMA	100% (30)	ND	100% (30)	ND	ND	ND
Paraguay	SDG	100% (30)	ND	ND	ND	ND	ND
	ASU	100% (30)	ND	96.7% (30)	ND	ND	ND
Uruguay	SAL	100% (30)	ND	100% (30)	ND	ND	ND
Argentina	MIA	ND	60% (30)	ND	66.7% (26)	ND	93.3% (30)
	ACO	100% (30)	ND	100% (30)	ND	ND	ND
	BUE	100% (30)	ND	96.6% (29)	ND	96.9% (33)	ND

Dissemination efficiency corresponds to the proportion of mosquitoes with disseminated virus in heads among tested ones. Numbers of analyzed mosquitoes are shown in parenthesis. The titer of infectious blood-meals was $10^{7.5}$ pfu/mL.
AE: *Aedes aegypti;* **AL**: *Aedes albopictus;* Viral strains: CHIKV_0621 isolated from La Réunion (ECSA genotype, E1-226V and E1-98A substitutions), CHIKV_115 isolated from La Réunion (ECSA genotype, E1-226A and E1-98A substitutions) and CHIKV_NC isolated from New Caledonia (Asian genotype, E1-226A and E1-98T substitutions). Mosquito populations (from North to South): **TYS** Tyson (United States), **VRB** Vero Beach (United States), **MXC** Chiapas (Mexico), **PAN** Panamá (Panama), **DEL** Delta Amacuro (Venezuela), **TUM** Tumbes (Peru), **PUM** Punchana (Peru), **MAN** Manaus (Brazil), **STR** Santarém (Brazil), **PNM** Parnamirim (Brazil), **CAB** Campos Belos (Brazil), **CPG** Campo Grande (Brazil), **JRB** Jurujuba (Brazil), **PAQ** Paquetá (Brazil), **VAZ** Vaz Lobo (Brazil), **BEL** Belford Roxo (Brazil), **SAN** Santos (Brazil), **BMA** Monteagudo (Bolivia), **SDG** Salto del Guairá (Paraguay), **ASU** Asuncion (Paraguay), **SAL** Salto (Uruguay), **MIA** Misiones (Argentina), **ACO** Corrientes (Argentina), **BUE** Buenos Aires (Argentina). ND: Not determined.
* Statistical differences of DE between the two mosquito species for a given virus ($P<0.05$)

Table 3. Dissemination efficiency of three CHIKV isolates in 22 *Ae. aegypti* and 13 *Ae. albopictus* populations from 10 American countries at day 10 post-infection.

Country	Mosquito population	CHIKV_0621		CHIKV_115		CHIKV_NC	
		AE	AL	AE	AL	AE	AL
United States	TYS	ND	93.3% (30)	ND	63.6%(11)	ND	ND
	VRB	100% (30)	85.7% (7)*	ND	96.7% (30)	ND	ND
Mexico	MXC	93.3% (30)	70.0% (30)*	100% (30)	53.3% (30)***	ND	ND
Panama	PAN	100% (30)	96.7% (30)	96.7% (30)	83.3% (30)	100% (30)	96.7% (30)
Venezuela	DEL	100% (10)	ND	100% (15)	ND	ND	ND
Peru	TUM	100% (30)	ND	ND	ND	ND	ND
	PUM	100% (29)	ND	100% (30)	ND	ND	ND
Brazil	MAN	100% (30)	100% (36)	ND	97.1% (34)	100% (30)	93.3% (30)
	STR	100%(30)	100% (20)	ND	ND	ND	ND
	PNM	100% (30)	90% (30)	ND	ND	ND	ND
	CAB	100% (30)	ND	ND	ND	ND	ND
	CPG	100% (30)	ND	100% (29)	ND	ND	ND
	JRB	100% (30)	100% (30)	100% (30)	ND	ND	ND
	PAQ	100% (30)	87.5% (32)*	100% (30)	ND	ND	ND
	VAZ	96.7% (30)	100% (32)	ND	ND	ND	ND
	BEL	100% (30)	88.9%(27)	ND	ND	ND	ND
	SAN	100% (29)	100% (30)	ND	ND	ND	ND
Bolivia	BMA	100% (30)	ND	100% (30)	ND	ND	ND
Paraguay	SDG	100% (30)	ND	ND	ND	ND	ND
	ASU	100% (30)	ND	93.3% (30)	ND	ND	ND
Uruguay	SAL	100% (30)	ND	100% (30)	ND	ND	ND
Argentina	MIA	ND	93.3% (30)	ND	80% (30)	ND	96.7% (30)
	ACO	100% (30)	ND	96.7% (30)	ND	ND	ND
	BUE	96.7% (30)	ND	100% (30)	ND	90% (30)	ND

Dissemination efficiency corresponds to the proportion of mosquitoes with disseminated virus in heads among tested ones. Numbers of analyzed mosquitoes are shown in parenthesis. **AE**: *Aedes aegypti;* **AL**: *Aedes albopictus;* Viral strains: CHIKV_0621 isolated from La Réunion (ECSA genotype, E1-226V and E1-98A substitutions), CHIKV_115 isolated from La Réunion (ECSA genotype, E1-226A and E1-98A substitutions) and CHIKV_NC isolated from New Caledonia (Asian genotype, E1-226A and E1-98T substitutions). Mosquito populations (from North to South): **TYS** Tyson (United States), **VRB** Vero Beach (United States), **MXC** Chiapas (Mexico), **PAN** Panamá (Panama), **DEL** Delta Amacuro (Venezuela), **TUM** Tumbes (Peru), **PUM** Punchana (Peru), **MAN** Manaus (Brazil), **STR** Santarém (Brazil), **PNM** Parnamirim (Brazil), **CAB** Campos Belos (Brazil), **CPG** Campo Grande (Brazil), **JRB** Jurujuba (Brazil), **PAQ** Paquetá (Brazil), **VAZ** Vaz Lobo (Brazil), **BEL** Belford Roxo (Brazil), **SAN** Santos (Brazil), **BMA** Monteagudo (Bolivia), **SDG** Salto del Guairá (Paraguay), **ASU** Asuncion (Paraguay), **SAL** Salto (Uruguay), **MIA** Misiones (Argentina), **ACO** Corrientes (Argentina), **BUE** Buenos Aires (Argentina). ND: Not determined.

* Statistical differences of DE between the two mosquito species for a given virus: * ($P<0.05$); *** ($P<0.001$).