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1 Impact of genetic diversity on biological characteristics of Usutu virus strains in Africa

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26 **Abstract:** Usutu virus (USUV) previously restricted to Africa where it caused mild infections,
27 emerged in 2001 in Europe and caused more severe infections among birds and humans with
28 neurological forms, suggesting an adaptation and increasing virulence. This evolution suggests
29 the need to better understand USUV transmission patterns for assessing risks and to develop
30 control strategies. Phylogenetic analysis conducted in Africa showed low genetic diversity of
31 African USUV strains except for one human and the USUV subtype (USUVsub) strains, which
32 exhibited a deletion in the 3'UTR and nucleotide substitutions throughout the genome. Here we
33 analyzed their viral replication *in vitro* in mosquito and mammalian cells, and vector competence

34 of *Culex quinquefasciatus*, compared to a reference strain. Growth kinetics of the different
35 strains showed comparable replication rates however variations in replication and translation
36 efficiency were observed. Vector competence analysis showed that all strains were able to infect
37 *Culex quinquefasciatus* the main peridomestic *Culex* species in Africa, with detection of USUV
38 viral genomes and infectious particles. Dissemination and transmission were observed only for
39 USUVsub, but infectious particles were not detected in *Culex quinquefasciatus* saliva. Our
40 findings suggest that genetic variability can affect USUV *in vitro* replication in a cell type-
41 dependent manner and *in vivo* in mosquitoes. In addition, the results show that *Culex*
42 *quinquefasciatus* is not competent for the USUV strains analyzed here and also suggest an
43 aborted transmission process for the USUVsub, which requires further investigations.

44

45 **Keywords:** Usutu virus, genetic diversity, *in vitro* viral growth, Vector competence and *Culex*
46 *quinquefasciatus*

47

48 **1. Introduction**

49 Usutu virus (USUV) is a member of the Japanese encephalitis serocomplex of the *Flaviviridae*
50 family isolated for the first time in 1959 in South Africa from a *Culex neavei* mosquito [Woodall
51 et al., 1963; McIntosh 1985; Poidinger et al., 1996]. USUV was reported in several African
52 countries mainly in mosquitoes and birds [Nikolay et al., 2011]. The virus was first recognized in
53 Europe in 2001 in association with the deaths of blackbirds (*Turdus merula*) and great grey owls
54 (*Strix nebulosa*) in Austria [Weissenböck et al., 2002]. However, a retrospective study on
55 paraffin-embedded tissues from dead birds found in Italy in 1996, showed detection of USUV
56 and suggested therefore that introduction of USUV in Europe occurred prior to 2001

57 [Weissenböck et al., 2013]. USUV has since been reported in several European countries
58 [Nikolay et al., 2012; Steinmetz et al., 2011].

59 The natural transmission cycle of USUV involves mosquitoes primarily of the *Culex* (*Cx.*) genus
60 and birds as amplifying hosts [Weissenböck et al., 2003; Brugger et al., 2009]. The virus was
61 detected in the wild from different mosquito species, in Senegal mainly from *Cx. neavei*
62 [Nikolay et al., 2011], in Ivory Coast from *Cx. quinquefasciatus* [Institut Pasteur de Dakar, IPD,
63 unpublished data] and in Kenya and Europe from *Cx. pipiens* [Ochieng et al., 2013; Chvala-
64 Mannsberger et al., 2007]. Vector competence studies showed that sylvatic species, *Cx. neavei* in
65 Africa [Nikolay et al., 2012] and domestic species, *Cx. pipiens* in Europe [Fros et al., 2015] were
66 able to transmit USUV.

67 Humans and other mammals such as horses, bats, dogs and wild boars can be accidental hosts
68 [Lelli et al., 2008; Cadar et al., 2013; Escribano-Romero et al., 2015]. In Africa, two mild cases
69 of human infections were reported in the Central African Republic (1981) and Burkina Faso
70 (2004) [IPD, unpublished data; Nikolay et al., 2011]. In Europe, two severe cases of
71 neuroinvasive infections in immunocompromised patients in Italy, due to USUV, were reported
72 for the first time in 2009 [Cavrini et al., 2009; Pecorari et al., 2009]. Since then, USUV specific
73 IgG were detected in blood donors from Italy [Cavrini et al., 2011; Gaibani et al., 2012;
74 Percivalle et al., 2017], and Germany [Allering et al., 2012; Cadar et al., 2017]. In 2013, three
75 patients with neuroinvasive symptoms were also diagnosed with USUV infection in Croatia
76 [Santini et al., 2015]. More recently, a retrospective analysis of patient material in Italy detected
77 USUV RNA in serum as well as in cerebrospinal fluid and USUV neutralizing antibodies in
78 serum [Grottola et al., 2017]. Another retrospective study in Montpellier showed USUV in the
79 cerebrospinal fluid of a patient with a clinical diagnosis of idiopathic facial paralysis [Simonin et

80 al., 2018]. In addition, USUV was detected in human blood donors in Austria in 2017 [Bakonyi
81 et al., 2017; Domanović et al., 2019] and 2018 [Aberle et al., 2018; Domanović et al., 2019], in
82 Germany in 2016 [Cadar et al.; 2017; Domanović et al., 2019], in Italy in 2017-2018 [Carletti et
83 al., 2019; Domanović et al., 2019]. All these data confirm USUV circulation in humans in
84 Europe and its neuroinvasiveness properties.

85 To understand the different epidemiological patterns between Africa and Europe, complete
86 genome sequencing and phylogenetic analyses of African and European strains were done. These
87 analyses showed overall very limited genetic diversity among all USUV strains analyzed
88 [Nikolay et al., 2013a]. However, a subtype of USUV (USUVsub), with a large number of
89 substitutions throughout the genome was identified and corresponds to isolate ArB1803 isolated
90 in 1969 from *Culex perfuscus* in Central African Republic (CAR). In addition, another strain
91 isolated from a human in 1981 in CAR was also identified with mutations at the 3' non-coding
92 region [Nikolay et al., 2013a].

93 USUV therefore shows limited genetic variations and geographical distribution (only in Africa
94 and Europe) with a seemingly minor impact on public health. However, migratory birds might
95 lead to the propagation of the virus, as seen for West Nile virus, and other members of the
96 Japanese encephalitis serocomplex. In addition, the increasing detection and virulence in Europe
97 suggested that USUV is becoming an emerging pathogen [Grottola et al., 2017] with potential
98 for global emergence.

99 For a better understanding of the transmission dynamics and preparedness against global
100 emergence risk, the African USUV strains should be better characterized and the urban vectors
101 capable of transmitting the virus to humans identified. Regarding the transmission of USUV to
102 humans, *Cx. quinquefasciatus* seems to be the main candidate in the West African context

103 regarding its presence all year round, in the domestic environment and in interaction with human
104 populations [Gowda et al., 1992]. In addition, *Cx. pipiens*, which is a member of the *Cx.*
105 *quinquefasciatus* complex, is known to be the main vector of USUV in Europe [Chvala-
106 Mannsberger et al., 2007].

107 In this regard, we analyzed here the viral replication *in vitro* and the vector competence of
108 peridomestic mosquito *Cx. quinquefasciatus* for different USUV strains. The impact of genetic
109 diversity between these USUV strains on viral growth and vector competence was also analyzed.
110 Because the existing USUV specific real-time RT-PCR was not able to detect the USUVsub
111 [Nikolay et al., 2013b], we developed a specific USUVsub RT-PCR assay in this study.

112 **2. Materials and Methods**

113 **2.1 Virus strains**

114 The USUV strains analyzed in this study were provided by the *Institut Pasteur de Dakar* (IPD),
115 WHO Collaborating Center for arboviruses and viral hemorrhagic fevers (CRORA) in Senegal
116 and are described in Table 1. Human strain (HB81P08) and USUVsub (ArB1803), which
117 exhibited highest genetic variations [Nikolay et al., 2013a] were analyzed in comparison to the
118 reference strain (SAAR1776).

119 **2.2 Cells lines**

120 Three cells lines were used for viral stock preparation (C6-36 cells (*Aedes albopictus*)), viral
121 stock titration (PS cells (Porcine Stable kidney cells, ATCC number, Manassas, USA) and
122 growth kinetics (C6/36, and VERO cells (Renal epithelial cells of *Cercopithecus aethiops*, Sigma
123 Aldrich, France)). These cell lines were grown with L15 medium containing 10% Foetal Bovine
124 Serum (FBS), 1% penicillin-streptomycin, and 0.05% fungizone for mammalian and plus 10%
125 tryptose phosphate for mosquito cells.

126 **2. 3 Suckling mice**

127 Mice were produced in the Institut Pasteur de Dakar farm, located in Mbao, approximately 15
128 kilometers from Dakar, Senegal. Newborn Swiss mice were placed in full-walled metal cages
129 with a mesh lid, and a lactating female. They received a cereal-based diet and water, with a
130 temperature between 22 and 24° C. These suckling mice from one to two days old were used for
131 viral isolation by intracranial infection.

132 **2. 4 Mosquitoes**

133 *Cx. quinquefasciatus* larvae were collected from a ground pool in Barkedji (15°17N, 14°53W), a
134 village in the northern Sahelian region of Senegal. For the infection experiments, F1 generation
135 adult mosquitoes were reared in the laboratory by using standard methods with a temperature of
136 $27 \pm 1^\circ\text{C}$, a relative humidity of 70–75%, and a 12-hour photoperiod [Gerberg et al., 1994].

137 **2.5 Viral stock preparation**

138 For *in vitro* kinetic experiments viral stocks were prepared by infecting C6-36 cells with the
139 different USUV strains (Table 1) for 4 days. To assess the cells infection by USUV,
140 immunofluorescence assay (IFA) was done as described previously [Digoutte et al., 1992,
141 Nikolay 2012]. Briefly, cells were dissolved in PBS and dropped on a glass slide. After complete
142 drying, cells were fixed in cold acetone, dried again, and then stored at -20°C until staining.
143 Staining was done with a USUV-specific polyclonal mouse immune ascit (polyclonal mouse
144 immune ascites produced with the whole inactivated USUV reference strain) diluted in PBS1X
145 as first antibody. Then cells were incubated with the second antibody (1/40 goat anti-mouse IgG,
146 1/100 Evan's blue, diluted in PBS1X). Examination was done by fluorescence microscopy.

147 For vector competence analysis, viral stocks were prepared by intracerebral infection of suckling
148 mice in order to reach high viral titers. Five days after the inoculation, the mice presented

149 symptoms of infection and the brains were recovered and homogenized into L15 medium. The
150 presence of USUV in the brain homogenates was tested by reverse transcription - quantitative
151 polymerase chain reaction (RT-PCR) as previously described [Nikolay et al., 2012].

152 The different viral stocks (from cells or suckling mice) were aliquoted and frozen at -80°C for
153 further experiments. For growth kinetics and mosquito infection experiments viral stocks were
154 titrated as previously described, using PS cells [De Madrid et Porterfield 1969; Nikolay et al.,
155 2012; Fall et al., 2014]. The plaque sizes of the different strains were also analyzed.

156 **2.6 Growth kinetics**

157 The growth kinetics were done as previously described [Stock et al., 2013; Fall et al., 2017].
158 Briefly, mammalian VERO and mosquito C6-36 cells in culture were infected in 12-well plate (1
159 plate for 1 strain) with a multiplicity of infection (MOI) of 0.01. For each plate, supernatant and
160 cells were harvested after 22, 28, 50, 75, 99, 124, and 146 hours post infection. Supernatants
161 were analyzed by titration and RT-PCR and the cells by IFA as previously described [Stock et
162 al., 2013; Fall et al., 2017]. The cells were analyzed by IFA assays to estimate the production of
163 viral antigens and RT-PCR assays to measure the viral RNA replication inside the cells while the
164 supernatants were analyzed by RT-PCR to estimate the number of viral particles released, and by
165 titration to measure the number of infectious particles. Finally, we estimated the replication
166 efficiency by calculating the ratio of the number of total released particles in the supernatant
167 divided by the number of plaque forming units (PFU), for each time point and cell line
168 [Weidmann et al., 2011].

169 Strain growth rates were compared using the R software (R version 3.3.2, *The R Foundation for*
170 *Statistical Computing*) using the Kruskal Wallis test, which permits to compare strains
171 replication in pairs at each sampling time (significant when p-value was less than 0.05).

172 **2.7 Oral infection of mosquitoes**

173 Oral infections were performed as already described [Nikolay et al., 2012; Fall et al., 2014;
174 Ndiaye et al., 2016]. Briefly, female mosquitoes were exposed to an infectious blood meal
175 containing the different USUV strains and the remaining blood meal was titrated. The
176 mosquitoes were then cold anesthetized and the engorged mosquitoes were selected and
177 incubated at 28°C, with relative humidity of 70–80% and fed with sucrose at 10% for 15 days. A
178 second oral infection was done when less than 30 mosquitoes were engorged during the first oral
179 infection. To follow the evolution of infection and dissemination over time, specimens were
180 collected and killed, frozen at 4, 8, and 12 days post-feeding (dpf). For each mosquito, both legs
181 and wings were placed in one tube and the body in another separate tube. At day 15 post-
182 infection, the remaining mosquitoes were collected and each mosquito was processed separately
183 to collect legs/wings, bodies and saliva as previously described [Nikolay et al., 2012; Fall et al.,
184 2014; Ndiaye et al., 2016]. All samples were stored at –80°C until testing.

185 **2.8 Analysis of mosquito samples**

186 Each mosquito sample was tested for the presence of USUV by RT-PCR and IFA. The bodies
187 were first screened by RT-PCR followed by legs and wings of mosquitoes with positive bodies,
188 and saliva when legs and wings were positive [Nikolay et al., 2012; Fall et al., 2014].

189 Viral isolation was done in C6-36 cells to show presence of infectious particles by IFA in RT-
190 PCR positive samples as well as to amplify low tittered samples. Negative samples were
191 passaged up to 3 times to confirm their negativity.

192 Samples were considered positive when they were detected by RT-PCR and confirmed by IFA.

193 The rates of infection (number of positive bodies/ number of tested mosquitoes), dissemination
194 (number of positive legs-wings/ number of positive bodies) and transmission (number of positive

195 saliva/ number of positive legs-wings) were compared using R software (R version 3.3.2). The
196 transmission rates estimated here by analyzing the positive saliva correspond to the potential or
197 transmissible mosquito infection rates.

198 **2.9 RNA extraction and real time RT-PCR**

199 Extraction of viral RNA from supernatants and cell suspension was performed with the QIAamp
200 viral RNA mini kit (Qiagen, Heiden, Germany) according to manufacturer's instructions. Cells
201 were lysed by serial cycles of freeze/thaw before RNA extraction.

202 For the detection and quantification of viral RNA, a consensus USUV real-time RT-PCR assay
203 and corresponding RNA standard targeting the NS5 gene was used for SAAR1776 and HB81P08
204 strains, as previously described [Nikolay et al., 2013b] (Table 2). This Usutu virus specific real-
205 time RT-PCR was not able to detect USUVsub [Nikolay et al., 2013b], we therefore additionally
206 developed specific set of primers and probe for USUVsub also targeting the NS5 sequence
207 (Table 2).

208 Both primers and probes systems were synthesised (TIB Mol-Biol, Berlin, Germany) and tested.
209 The real-time PCR assays were performed using the Quantitect Probe RT-PCR Kit (Qiagen,
210 Heiden, Germany) in a 96-well plate under the following conditions: 50°C for 15 min, 95°C for
211 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Copy numbers of genome
212 were calculated using Ct (Cycle threshold) and corresponding RNA standard.

213 **2.10 Development of USUVsub RT-PCR assay**

214 **2.10.1 Standard RNA for USUVsub**

215 Primers 1803 NS5 F1 (CCGAGGACAGGATGAACTCA) and 1803 NS5 R1
216 (TGGCCTGACATTCCTACT) (TIB Molbiol, Berlin, Germany) designed in this study were
217 used to amplify the NS5 gene (650bp) of the USUVsub. Reverse transcription was done using

218 the AMV kit (Invitrogen, Carlsbad USA) and the 1803 NS5 R1 primer, following the provider's
219 instructions. The resulting complementary DNA was amplified using Go-Taq PCR kit (Promega,
220 Madison, USA) and the PCR conditions are the following: 5 min 94 °C, 45cycles of 1 min 94 °C,
221 1 min 53 °C and 1 min 72°C, and 10 min 72°C. The RNA standard was synthesized at TIB
222 Molbiol with the PCR product obtained as previously described [Fall et al., 2016].

223 **2.10.2 Determination of analytical specificity and sensitivity**

224 Ten-fold dilutions of the RNA standard with known copy number were quantified in triplicate
225 using the USUVsub primers and probe. Regression curves were obtained representing the RNA
226 copy number/reaction vs the threshold cycle value (Ct). The lowest RNA copy number with RT-
227 PCR detection was considered as the analytical detection limit.

228 In parallel, ten-fold dilutions in L15-medium of a viral stock of USUVsub with a known viral
229 infectious titer was similarly quantified in triplicate and the lowest number of infectious virus
230 particles with RT-PCR detection was considered as the analytical detection limit in serum.

231 The specificity of the assay was determined by testing other USUV strains, and flaviviruses West
232 Nile, Zika, yellow fever and dengue strains (Table 3). The amplification efficiency of the primers
233 was calculated from the slope of the standard regression lines ($E=10^{1/\text{slope}} - 1$).

234

235 **2.11 Secondary structure analysis**

236 VISUALOMP version 7 and FORNA (force-directed RNA) were used to predict and fold the
237 RNA secondary structures at 37°C and 20°C [Kerpedjiev et al., 2015].

238 **3. Results**

239 **3.1 Viral stocks**

240 For *in vitro* kinetic experiments, the viral stocks were prepared in C6/36 cells and the following
241 titers were observed: 4.25×10^7 pfu/ml, 2×10^4 pfu/ml and 3×10^5 pfu/ml, respectively for
242 SAAR1776, HB81P08 and ArB1803.

243 For mosquito infections, higher viral titers were needed and the viral stocks were prepared in
244 suckling mice. The following higher titers were observed: 3.5×10^{10} pfu/ml, 3×10^{10} pfu/ml and
245 1.35×10^9 pfu/ml, respectively for SAAR1776, HB81P08 and ArB1803.

246 The USUVsub showed small plaques size while other USUV strains showed greater plaques in
247 PS cells with viral stocks prepared both in C6/36 cells and in suckling mice (Figure 1).

248 **3.2 Validation of USUV subtype RT-PCR assay**

249 RNA from different USUV and other flavivirus strains had been previously tested and
250 successfully detected by Pan-Flavi primers and probe RT-PCR assay [Patel et al., 2013]. The
251 USUVsub RT-PCR assay did not detect other USUV strains or cross-detect other flaviviruses
252 like yellow fever, dengue, West Nile and Zika viruses (Table 3).

253 The analytical detection limit of the RT-PCR assay tested with the RNA standard was 100
254 copies/ reaction. In addition, the detection limit was tested with the viral stock in L-15 medium
255 and was 45 pfu/ reaction. Efficiencies ranged from 91 to 94.5% (Figure 2).

256 **3.3 Growth kinetics**

257 In Vero cells, regarding the intra-cellular replication, all the strains had comparable genome
258 replication (*p-values*= 0.12-0.8) (Figure 3, panel A) while variations were observed for the
259 antigen production (Figure 3, panel D). Indeed, the reference and human strains had greater
260 antigen production rates and the USUVsub had lower rates from 99 to 146 hpi (*p-values* =0.03-
261 0.04). Analyses of supernatants showed statistically comparable total released (Figure 3, panel
262 B) and infectious particles (Figure 3, panel C) for all strains. The ratios of genome copy number /

263 infectious virions (pfu) showed that in mammalian cells, the reference and the human strains
264 presented lower ratios, producing about as many genome copies as infectious particles, while
265 USUVsub showed higher ratios showing overproduction of genome copies (p -values= 0.04)
266 (Figure 4, panel A).

267 In C6/36 cells, regarding the intra-cellular replication, the USUVsub showed significant
268 differences in the genome replication and antigen production (Figure 3, panels E and H). Indeed,
269 the USUVsub led to higher genome copy numbers from 28 to 50h pi (p -values= 0.04) and lower
270 genome copy numbers from 99 to 126h pi (p -values= 0.04). The antigen production was
271 comparable for all strains except at 75 and 99h pi where USUVsub showed lower production
272 rates (p -values= 0.04). Analyses of supernatants showed also that all strains had statistically
273 comparable total released (Figure 3, panel F) and infectious particles (Figure 3, panel G) (p -
274 values > 0.05). In mosquito cells, the ratio of genome copy number / infectious virions (pfu) for
275 all strains were comparable (p -values= 0.12 - 0.82) (Figure 4).

276 The USUVsub developed much smaller plaques in mammalian PS cells compared to other
277 strains, similar to the original viral stock plaque sizes (Figure 1).

278 **3.4 Vector competence of *Cx. quinquefasciatus* mosquitoes**

279 For the HB81P08 strain, one infection was done and the viral titer post feeding was (pfe) $4.5 \times$
280 10^7 pfu/ml. RT-PCR tests detected infection rates of 50, 16.66 and 40% for 8, 12 and 15 dpi
281 respectively. IFA tests confirmed the infection rates for 8 and 12 dpi while at 15 dpi only 33.33%
282 of the samples were confirmed. No virus was detected in the legs/wings with both methods
283 (Figure 5, column 3).

284 One experimental infection was done for strain SAAR1776, and the blood meal titer post feeding
285 (pfe) was 5×10^6 pfu/ml. RT-PCRs tests detected infection rates of 40, 50, 60 and 44.61% for 4, 8,

286 12 and 15 dpi respectively (Figure 5, column 4). IFA tests did not confirm infection at 4 dpi
287 while 40, 20 and 18.46% of tested samples were confirmed at 8, 12, and 15 dpi respectively. For
288 the dissemination, RT-PCRs tests of the legs/wings gave rates of 20%, 0% and 20.68% at 8, 12,
289 and 15 dpi (Figure 5). In contrast, IFA showed negative results for all the RT-PCR positive
290 legs/wings and did not confirm viral dissemination (Figure 5).

291 For USUVsub (ArB1803), two experimental infections were done; the blood meal contained
292 5.75×10^7 and 3.25×10^6 pfu/ml. RT-PCRs tests detected infection rates between 40 to 50% at 4, 8
293 and 12 dpi for experiment 2. Infection rates were 19.51% and 38.70% at 15 dpi for experiments 1
294 and 2 respectively (Figure 5, columns A and B). However, IFA tests confirmed only 5 % of
295 infection at 4 dpi and 20% at 8 and 12 dpi. For 15 dpi, 15.85% and 29.03% were confirmed
296 respectively for both infections (Figure 5, upper panels columns A and B). Dissemination was
297 shown for this strain by RT-PCR with rates of 30 and 44.44% at 4 and 8 dpi. The virus was not
298 detected in the mosquito's legs-wings at 12 dpi. Dissemination rates were 58.33% and 18.75% at
299 15dpi for experiments 1 and 2 respectively (Figure 5, middle panels columns A and B). IFA tests
300 confirmed dissemination only at 15 dpi with rates of 7.69% and 55.55% for both experiments
301 (Figure 5). Transmission rates estimated by RT-PCR tests were 42.85% and 33.33% at 15 dpi for
302 both experiments (Figure 5, lower panels columns A and B). The corresponding viral genome
303 copy numbers in these positive salivas were determined using the RNA standard developed in
304 this study and ranged between 1042 to 3232 per ml. However, no infectious particles were
305 detected in the saliva samples by IFA and therefore the transmission of the USUVsub was not
306 confirmed.

307 The statistic tests for equality of proportions showed no difference in the infection capabilities of
308 reference, human and USUVsub strains (SAAR1776, HB81P08, and ArB1803) by RT-PCR as
309 well as by with p-values of 0.88 and 0.80 respectively.

310 **4. Discussion**

311 Here we investigated growth behavior *in vitro* in mosquito (C6/36) and primate (Vero) cell lines
312 as well as *in vivo* in *Cx. quinquefasciatus* of 3 distinct USUV strains. We chose primate and
313 mosquito cell lines to mimic vector and vertebrate hosts in the natural life cycle of USUV. We
314 found that genetic differences, as well as viral-mosquito interactions, probably play a role in the
315 biological properties such as: (i) genome replication, (ii) protein translation, and (iii)
316 susceptibility to infect and disseminate in mosquitoes.

317 We first developed a real time RT-PCR assay for USUVsub detection. The analytical sensitivity
318 of the previously described USUV assay (60 copies/ reaction) was comparable to the sensitivity
319 of the USUVsub assay developed in this study (100 copies/ reaction) [Nikolay et al., 2013b]. For
320 the detection of corresponding viral particles, the detection limit of the RT-PCR assay was 1.2
321 pfu/ reaction with the USUV assay and 45 pfu/ reaction for the USUVsub assay [Nikolay et al.,
322 2013b]. In addition, the USUVsub assay showed good specificity and was not able to detect
323 other USUV or flavivirus strains.

324 Analyzing RT-PCR and plaque assay results, USUVsub appeared to overproduce genomes
325 (Figure 4A) resulting in viral particles with reduced infectivity, indicating defective particles due
326 to less efficient packaging [Weidmann et al., 2011]. While the reference and the human strains
327 were more efficient in producing infectious particles (Figure 3C). The infectious particles
328 produced by USUVsub were down at least 1 log in comparison to the other two strains. In
329 contrast, in mosquito cells, all strains showed overall comparable replication efficiency.

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The human and the reference USUV strains showed similar replication rates in both cell lines, meaning that the mutations at the 3' non-coding region of the human strain did not impact on its replication *in vitro* with the cell lines used for this study.

A phylogenetic study done by Nikolay and colleagues revealed that the NS5 protein, which has RNA-dependent polymerase and methyltransferase activity [Danecek et al., 2010], was the most conserved region of USUV strains [Nikolay et al., 2013a]. This could explain the comparable replication rates for human and reference USUV strains. Similar results were also observed for different West Nile virus strains in mammalian (VERO) and mosquito (AP61) cells [Fall et al., 2017]. For USUVsub, substitutions located in the NS5 protein and also in other genome regions led probably to the observed variations cell type-dependent [Nikolay et al., 2013a]. Indeed, in C6/36 cells, minor replication rate variations were detected while in mammalian cells, delay on protein translation was clearly measurable for USUVsub. In addition, this strain showed lower lysis plaque sizes during viral titrations on mammalian PS cells irrespective of previous culture in mice or on C6/36 cells. Recently it was shown that Zika virus strains isolated from humans display large plaques on mammalian cells and small plaques on C6/36 cells, which was seen as a host effect i.e. essentially adaptation to the host from which they were isolated (Moser et al 2018). Similarly USUVsub originally isolated from the mosquito *Culex perfuscus* did produce only small plaques on mammalian PS cells. This correlates quite well with the out of sync production of a low number of infectious particles of USUVsub in mammalian Vero cells (Fig. 4A, Fig 3C) and clearly indicates inefficient replication in mammalian cells of this mosquito isolate.

353 Reference and human strains have already been characterized in mice and results showed
354 comparable mortality when applied by the intracerebral route, however in intraperitoneal and
355 subcutaneous routes, the reference strain showed higher virulence and mortality [Diagne et al.,
356 2019]. These data suggest that depending on the infection route, the mutations at the 3' non-
357 coding region of the human strain had a negative impact on its replication and virulence *in vivo*
358 in mice. Therefore, further studies with mice models are needed to better explore and understand
359 the virulence of the USUVsub compared to the other strains.

360 We also performed a vector competence study of *Cx. quinquefasciatus*, an anthropophilic and
361 competent peridomestic vector for West Nile and Rift Valley fever viruses in Africa [Fall et al.,
362 2014; Ndiaye et al., 2016], in order to better understand the USUV transmission cycle. Our result
363 showed that *Cx. quinquefasciatus* is susceptible to all USUV strains analyzed, while
364 dissemination in the mosquito legs and wings was observed only for USUVsub. In mosquito
365 saliva, we were able to detect viral RNA for USUVsub only, however, no infectious viral
366 particles were found. These results demonstrate that *Culex quinquefasciatus* from Senegal was
367 not able to transmit the USUV strains analyzed here.

368 In Senegal, Nikolay *and colleagues* [Nikolay et al., 2012] showed that the mosquito *Cx. neavei*
369 was able to transmit the USUV reference strain using a blood meal titer, which did not exceed
370 4.5×10^6 pfu/ml. In our study we infected *Cx. quinquefasciatus* mosquitoes with the same strain
371 with 5×10^6 pfu/ml and for the others strains even higher viral titers (HB81P08: 4.5×10^7 pfu/ml,
372 ArB1803: 3.25×10^6 to 5.75×10^7 pfu/ml) were used. In Europe, to investigate vector competence
373 of *Cx. pipiens*, belonging to the same complex as *Cx. quinquefasciatus*, Fros *and colleagues*
374 performed their oral infection with 50% tissue culture infectious dose (TCID₅₀) of 4×10^7 per ml
375 [Fros et al., 2015] and for *Aedes albopictus* $0.66 \times 10^{7.5}$ / $0.66 \times 10^{7.9}$ TCID₅₀/ml were used

376 [Puggioli et al., 2017]. All these studies used viral titers comparable to those obtained in our
377 study, so the viral titers used did not affect their transmission by *Cx. quinquefasciatus*.

378 The human and reference strains had similar infection profiles, we therefore assumed that the
379 mutations at the 3' non-coding region of the human strain did not impact its replication in
380 mosquitoes.

381 Although there was no transmission, USUVsub seemed to be more adapted to *Cx*
382 *quinquefasciatus* than the other strains. Indeed, USUVsub infectious particles were detected in
383 mosquito bodies and legs/wings while for other strains, the infectious particles were limited to
384 the mosquito bodies even if viral RNA was detected in legs/wings. These differences in the
385 mosquito infection patterns could be explained by the genetic variability of the virus strains used
386 in our experiment. The numerous substitutions observed in the USUVsub genome might increase
387 its fitness in *Cx. quinquefasciatus*. Similar studies done with West Nile virus also showed the
388 impact of genetic variability on *Cx. quinquefasciatus* infection patterns [Fall et al., 2014].

389 More studies could be done to better characterize this USUVsub genetic variability in order to
390 better understand the role and nature of genetic substitutions to mosquito infection. In recent
391 years the role of secondary structures in the 3'UTR of flavivirus genomes and the number and
392 length of subgenomic flavivirus RNAs (sfRNAs) coded for in this region have been shown to be
393 relevant for host specificity [Slonchak et al., 2018]. Differences in secondary structure have been
394 linked to adaptation and transmission by mosquitoes [Yeh et al., 2018; Villordo et al., 2015;
395 Moser et al., 2018]. Secondary structure analysis indicates that the predicted secondary structures
396 for the 3'UTR of USUVsub differ significantly from those described so far (Figure S1). This
397 difference may be related to the observed efficient replication of USUVsub in the mosquito cells
398 *in vivo* but needs further investigation beyond the scope of this study.

399 Combining RT-PCR and IFA showed that many viral particles produced during the mosquito
400 infection are defective (Figure 5). Indeed, all the viral particles detected by RT-PCR in the
401 different mosquito compartments, were not confirmed by IFA, this latter technique allowing the
402 detection of viral infectious particles. Our results suggest that the viral infection process of *Cx.*
403 *quinquefasciatus* with USUV strains was aborted and only defective viral particles were released
404 in mosquito legs/wings for the reference strain and into the saliva for USUVsub. This indicates
405 that there is no USUV transmission by *Cx. quinquefasciatus* and more studies are needed to
406 better understand the abortion of USUV viral infection in the legs/wings and saliva of *Cx.*
407 *quinquefasciatus*. These results highlight the need to include virus isolation and IFA in vector
408 competence analysis to prove that RNA detected by RT-PCR corresponds to infectious viral
409 particles that could replicate in a vertebrate host after transmission during a mosquito blood
410 meal.

411 In Senegal the circulation of USUV is monitored by entomological surveillance at Pasteur
412 Institute of Dakar, which showed a circulation of the virus mainly in *Cx neavei* species until
413 2016 [CRORA database, IPD unpublished data; Nikolay et al., 2011]. The virus has never been
414 isolated from *Cx quinquefasciatus* in the field, and experimentally we confirmed that this
415 mosquito species is not able to transmit USUV. Although the reference strain showed virulence
416 and induced mortality in vertebrate hosts [Gaibani et al., 2012, Diagne et al., 2019], the absence
417 of transmission by *Cx. quinquefasciatus* could explain the lack of USUV human cases in Senegal
418 and West Africa. However, vector competence studies with others mosquito species in Africa
419 should be done to better investigate the urban transmission of USUV.

420 **5. Conclusions**

421 The low genetic diversity described for USUV [Nikolay et al., 2013a] had a minor impact *in*
422 *vitro* and a significant impact *in vivo* in the mosquito *Cx. quinquefasciatus* even if this mosquito
423 species was not able to transmit the virus. Among the strains analyzed in this study, USUVsub
424 was the most divergent. Further complementary studies using mouse model would allow us to
425 better understand the pathogenicity of this strain.

426 As evidenced by Zika virus, the epidemiology of infectious diseases depends on climatic,
427 ecological and human related factors. Just 2 sporadic non-severe USUV cases in humans have
428 been described in Africa [Cavrini et al., 2009; Pecorari et al., 2009; Busani et al., Ochieng et al.,
429 2013; Cadar et al., 2017]. However, in Europe, severe cases of human infections have been
430 detected. Therefore, more vector competence studies for USUV are needed to identify competent
431 peridomestic vectors. In addition, entomological, animal reservoir and human surveillance need
432 to be strengthened to understand the level of circulation of this virus in Africa.

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Table 1: Strains used in this study

Three different USUV strains were used in this study. Geographic origins, year of isolation, host and accession numbers are indicated.

* Ap3/NBM3/C61 is equivalent to 3 serial passages in Ap61 (Ap) followed by 2 passages in newborn mice (NBM) followed by 1 passage in C6/36 (C6).

ISOLATE NAME	GEOGRAPHIC ORIGIN	YEAR	HOST	NUMBER OF PASSAGES	*PASSAGE HISTORY	ACCESSION NUMB
SAAR1776	South Africa	1959	<i>Culex neavei</i>	7	AP3/NBM3/C61	AY453412
ArB1803	Central African Republic	1969	<i>Culex perfuscus</i>	7	AP3/NBM3/C61	KC754958
HB81P08	Central African Republic	1981	Human	7	AP3/NBM3/C61	KC754955

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Table 2: Primers and probes used in this study

Primers and probes used in this study are indicated in this table. The USUV assay previously developed [Nikolay et al., 2013b] permits the detection of reference and human strains and the USUVsub assay developed in this study allows the detection of USUVsub.

Primers and probes	Sequences	Region
Usu FP (USUV)	5'- CAAAGCTGGACAGACATCCCTTAC	NS5
Usu RP (USUV)	5'- CGTAGATGTTTTTCAGCCCACGT	NS5
Usu P (USUV)	5'- 6FAM-AAGACATATGGTGTGGAAGCCTGATAGGCA--TMR	NS5
NF FP (USUVsub)	5'- AGAGCTGGACGGAAGTTCCCTA	NS5
NF RP (USUVsub)	5'- TCTCAGCCCATGTTGCACG	NS5
NF P (USUVsub)	5'- 6FAM-AAGAGAGAAGACATTTGGTGC GGCAGT--TMR	NS5
1803 NS5 F1 (USUVsub)	5'- CCGAGGACAGGATGAACTCA	NS5
1803 NS5 R1 (USUVsub)	5'- TGGCCTGACATTCCTACACT	NS5

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647 **Table 3: Specificity of the USUV subtype RT-PCR**

648 USUV and different flaviviruses strains were used to analyze the specificity of the USUVsub
 649 RT-PCR assay. The PanFlavi assay previously developed [Patel et al., 2013] were used to
 650 confirm presence of viral RNA in all the samples. The geographic origin, the host origin and the
 651 year of isolation of each strain were indicated in this table.

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Strains	Virus	Geographic origin	Host origin	Year of isolation	Panflavi primers	USUV Subtype primers
ArB1803	USUVsub	Central African Republic	<i>Culex perfuscus</i>	1969	31.55	21.74
SAAR1776	USUV	South Africa	<i>Culex neavei</i>	1959	26.17	-
HB81P08	USUV	Central African Republic	Human	1981	25.52	-
ArD101291	USUV	Senegal	<i>Culex gr. univittatus</i>	1993	24.67	-
259524	USUV	Senegal	<i>Mastomys natalensis</i>	2013	25.79	-
259520	USUV	Senegal	<i>Mastomys natalensis</i>	2013	25.83	-
FNV 281	Yellow fever	Ghana	Human	1927	20.46	-
New Guinea C	Dengue2	New Guinea	Human	1974	24.61	-
MR766	Zika	Uganda	Rhesus monkey	1947	28.02	-
B956	WNV	Uganda	Human	1937	32.91	-
Eg101	WNV	Egypt	Human	1951	35.69	-
ArD166362	WNV	Senegal	<i>Aedes vexans</i>	2002	37.41	-
Dak ArB209	Bagaza	Central African Republic	<i>Culex spp.</i>	1966	23.74	-
ArB490	Bouboui	Central African Republic	<i>Anopheles paludis</i>	1967	23.48	-
ArD14701	Kedougou	Senegal	<i>Aedes minutis</i>	1972	29.06	-
H177	Wesselsbron	South Africa	Human	1955	19.28	-

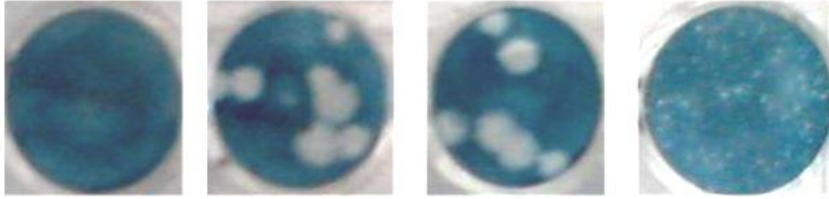
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Negative control

SAAR1776

HB81P08

ArB1803

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661 **Figure 1:** Titration of USUV strains. Shows the plaques obtained during USUV titration with PS

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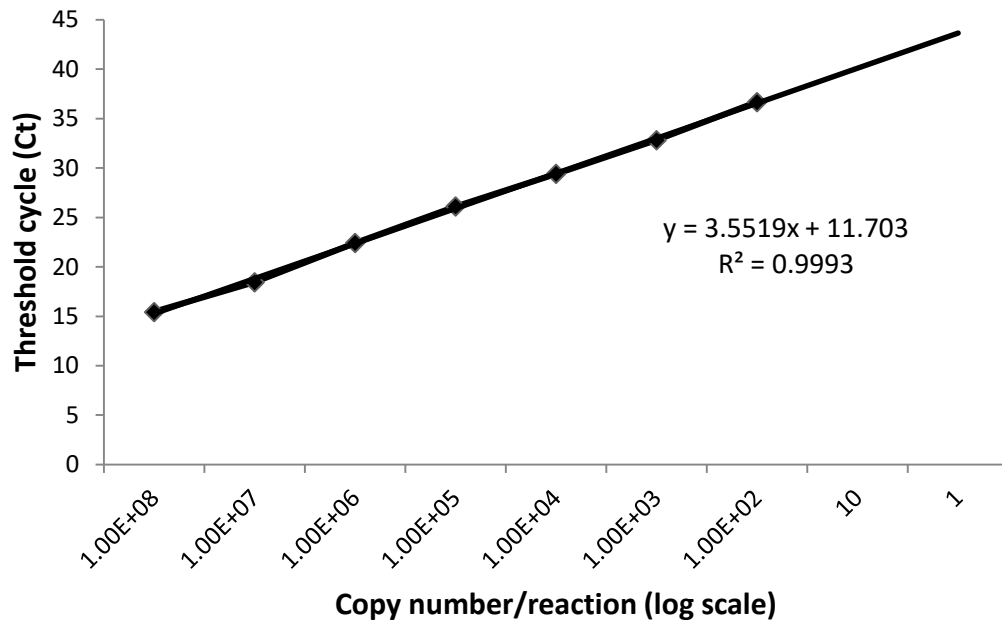
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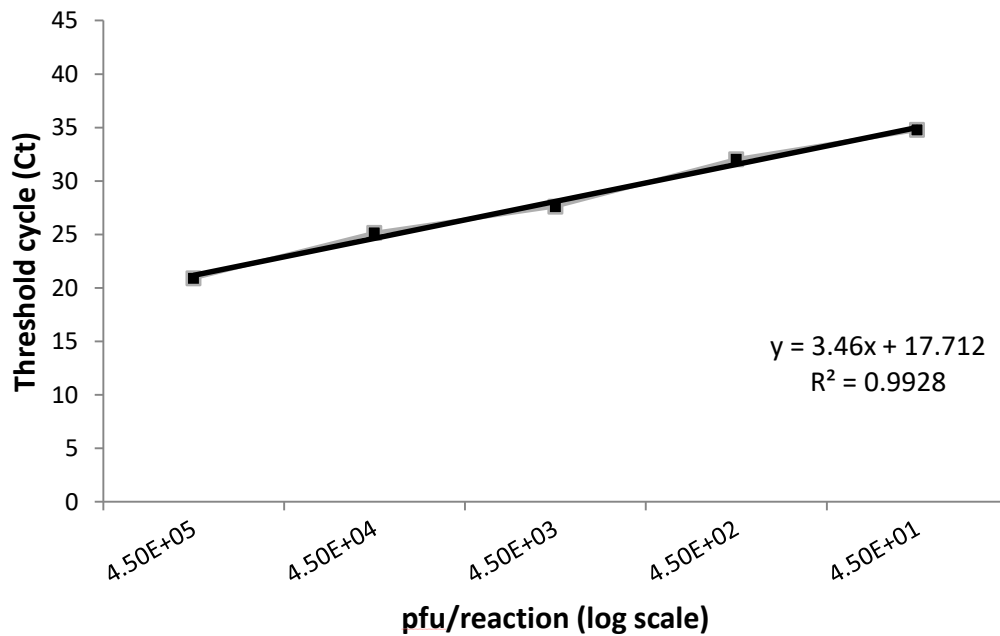
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685 A



686 B

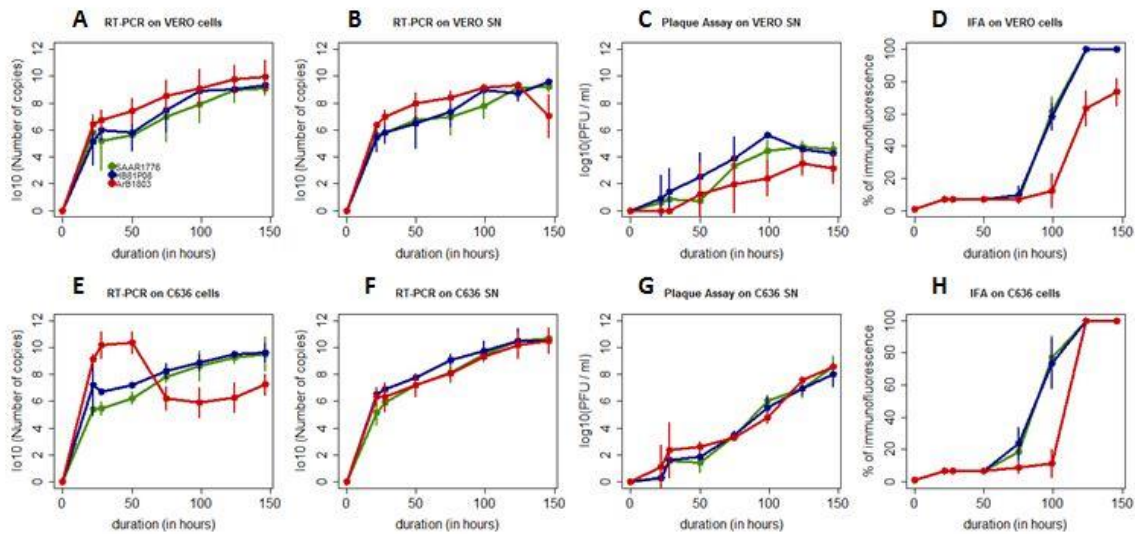


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689 **Figure 2: Sensitivity of the USUV subtype RT-PCR assay. (A):** Serial 10-fold dilutions of *in*
690 *vitro* RNA standard have been tested in the corresponding real-time RT-PCR assay. Tested
691 dilutions ranged from 1×10^8 to 1 copies/reaction for the RNA standard of the USUVsub specific
692 real-time RT-PCR assay. **(B):** Serial 10-fold dilutions of virus in L-15 medium have been tested

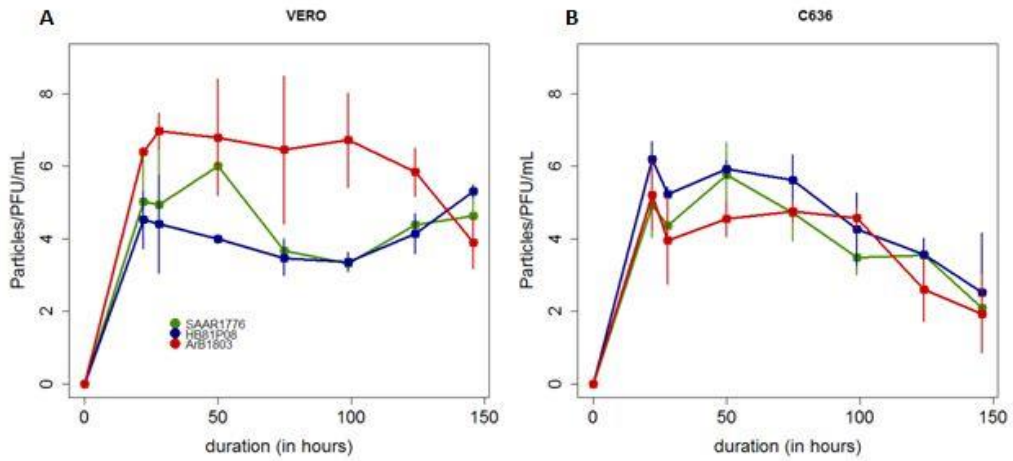
693 for USUVsub real-time RT-PCR assay. Tested dilutions ranged from 4.5×10^5 to 4.5 pfu for the
694 USUVsub viral stock.

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701 **Figure 3:** Growth kinetics of different strains of USUV mammalian (VERO) and in mosquito
702 (C6/36) cells. Amount of viral RNA equivalents isolated from cells (A and E) and from
703 supernatant (B and F) (\log_{10} of RNA copy number), the number of infectious viral particles (C
704 and G) (\log_{10} PFU/ml), and percentage of immunofluorescence of cells infected (D and H), and
705 at 22, 28, 50, 75, 99, 124 and 146 hours pi. The experiments were performed with C6/36 cells
706 (line below) and VERO cells (line above).

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711 **Figure 4:** Replication efficiency of USUV in mosquitoes (C6/36) and mammalian (VERO) cells.

712 Replication efficiency (Log particles/PFU/mL) of USUV strains in VERO (A) and C6/36 (B)

713 cell lines over 146-hour post-infection period.

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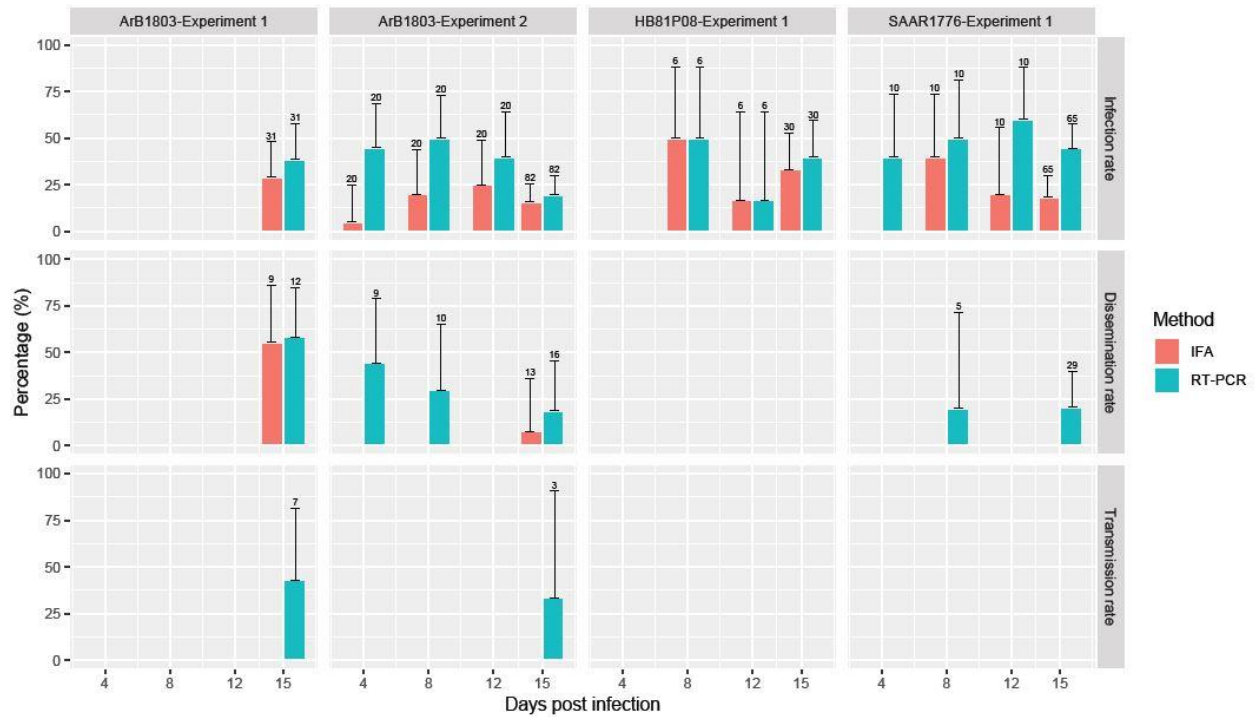
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724 **Figure 5:** qRT-PCR and Indirect Immunofluorescence Assay (IFA) of bodies, legs-wings and
725 saliva of mosquitoes infected with different strains of USUV.

726 Infection rates, dissemination rates and transmission rates of mosquitoes *Cx. quinquefasciatus*
727 infected with USUV strains at days 4, 8, 12 and 15 pi. All rates were estimated with RT-PCR
728 and IFA tests. The numbers above the bars represent the total number of individuals tested in
729 each day of sampling for each strain.

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