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Serosurvey of Chikungunya Virus in Old World Fruit Bats, Senegal, 2020–2022

Appendix

Materials and Methods

Bat blood samples collection

Bats were captured during July and December (the rainy season) using mist nets between dusk and dawn in five locations in the Kédougou region, Senegal. After capture, each bat was carefully restrained face-up in a cloth bag to minimize stress. Then, the blood sample was collected by cardiac puncture. Following sample collection, the bats were identified using standard ecologic taxonomic keys and released. Samples were stored in liquid nitrogen during the field to maintain sample integrity. Upon arrival at the laboratory, blood samples were centrifuged at 3,000 rpm for 15 minutes to separate the serum, and then the serum samples were stored at −80°C.

ELISA to detect antibodies targeting recombinant envelope protein 2 of Chikungunya virus

Our in-house ELISA protocol was based on previously established methods (*1*–*3*). Briefly, we coated 96-well plates (Cat no. #3690, Corning, USA) with 50 µL/well of a 2 µg/mL solution of chikungunya virus envelope protein 2 (E2) (Cat no. #MBS596329, MyBioSource, USA) in phosphate-buffered saline (PBS, Cat no. #10010–023, GIBCO, USA) overnight at 4°C. We removed the coating solution the next day and blocked the plates with 100 µL/well of 3% non-fat milk in PBS with 0.1% Tween 20 (PBST) for 1 hour at room temperature. To inactivate potential viruses, bat serum samples were heated at 56°C for 1 hour before use. Serial dilutions (1:100) of these serum samples and antibody controls were prepared in 1% non-fat milk PBST. Then, we removed the blocking solution and washed the plates three times with 250 µL/well of 0.1% PBST. Next, we added 100 µL of serial serum dilutions in each well and incubated the

plates for 1 hours at 37°C. Subsequently, the plates were washed three times with 250 µl/well of 0.1% PBST. For antibody detection, we used goat anti-bat IgG-horseradish peroxidase (HRP) conjugate (Cat no. #NB7238, Novus Biologicals, USA) diluted 1:10,000 in 0.1% PBST. We added 100 µL of this secondary antibody solution to each well and incubated for 1 hour. Positive and negative controls were included using a 1:10,000 dilution of goat anti-mouse IgG-HRP conjugate (Cat no. #SA5–10276, Thermo Fisher USA) prepared in the same manner. The plates were then washed three times with 0.1% PBST. After completely drying the plates, we added 100 µL of SIGMAFAST OPD (o-phenylenediamine dihydrochloride; Cat no. #P9187, Sigma-Aldrich, USA) substrate solution to each well. The reaction was allowed to proceed for 10 minutes before being stopped with 100 µl/well of 3M hydrochloric acid. The optical density (OD) was measured at 490 nm (OD490) using a Molecular Devices Versamax Microplate Reader. A sample was considered positive if its OD490 value exceeded the cutoff value, defined as the mean OD of negative controls (uninfected mice) plus three standard deviations. Additionally, we ran the control in the ELISA with only the secondary antibody (goat anti-bat IgG- HRP conjugate), and we did not observe any binding above the cutoff of the assay.

ELISA cross-reactivity assessment

To evaluate potential cross-reactivity between alphavirus antibodies and other arboviruses with the CHIKV-E2 recombinant protein, we screened polyclonal hyperimmune mouse fluid specific to o'nyong nyong virus, Semliki Forest virus, Venezuelan equine encephalitis virus (VEEV), yellow fever virus, and Uukuniemi virus obtained from the World Reference Center for Emerging Viruses and Arboviruses. Our in-house ELISA using the CHIKV-E2 recombinant protein detected antibodies against o'nyong nyong virus and Semliki Forest virus, suggesting cross-reactivity with these specific alphaviruses. No reactivity was observed with VEEV, yellow fever, or Uukuniemi virus.

References

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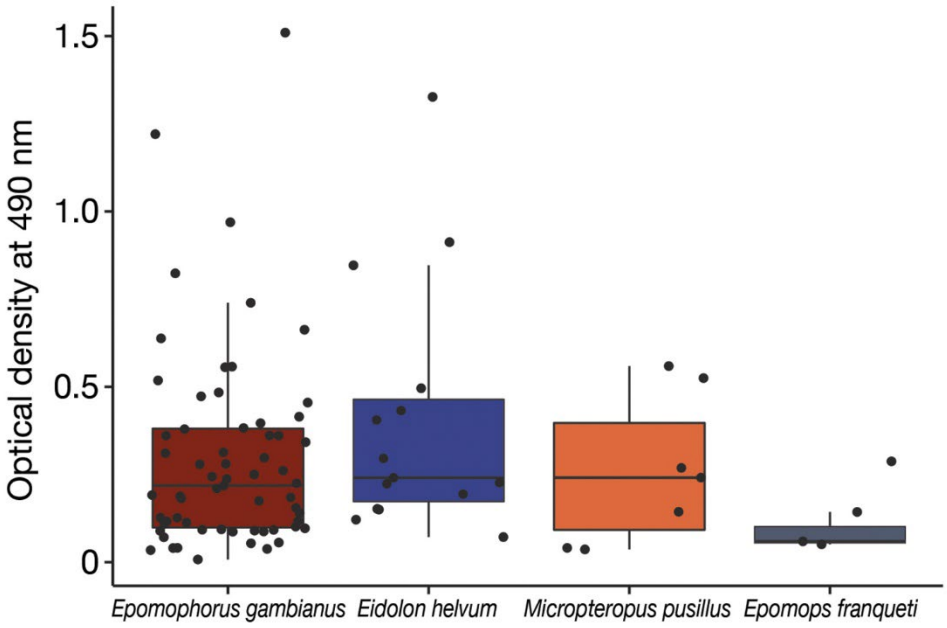
Appendix Table. Data from serosurvey of Chikungunya virus in Old World fruit bats, Senegal, 2020–2022, showing seropositive bat samples captured in Kédougou region.

Date	Location	GPS coordinates	ID	Species	Sex	CHIKV OD mean
26-Oct-2020	Fadiga	12°32′59″ N 12°11′55″ W	B13	*Epomophorus gambianus*	na	0.09225196
27-Oct-2020	Samecouta	12°36′46″ N 12°8′10″ W	B20	*E. gambianus*	F	0.27905196
27-Oct-2020	Samecouta	12°36′46″ N 12°8′10″ W	B21	*Epomops franqueti*	F	0.05105196
28-Oct-2020	Samecouta	12°36′46″ N 12°8′10″ W	B25	*Lissonycteris angolensis*	M	0.21305196
28-Oct-2020	Samecouta	12°36′46″ N 12°8′10″ W	B29	*Eidolon helvum*	M	0.22350196
30-Oct-2020	Ngari	12°38′5″ N 12°15′1″ W	B62	*E. gambianus*	F	0.09670196
24-Nov-2020	Fadiga	12°33′00.55″ N 12°11′55.17″ W	B90	*E. gambianus*	F	0.19160196
25-Nov-2020	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B99	*E. gambianus*	M	0.09285196
25-Nov-2020	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B101	*E. franqueti*	M	0.05950196
25-Nov-2020	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B105	*Micropteropus pusillus*	M	0.04080196
26-Nov-2020	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B111	*E. franqueti*	M	0.14320196
26-Nov-2020	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B112	*E. gambianus*	M	0.66280196
-Jun-2021	Samecouta	12°36′46″ N 12°8′10″ W	B130	*E. gambianus*	M	0.18490196
-Jun-2021	Samecouta	12°36′46″ N 12°8′10″ W	B135	*E. gambianus*	M	0.39630196
-Jun-2021	Samecouta	12°36′46″ N 12°8′10″ W	B144	*E. gambianus*	M	0.21855196
-Jun-2021	Samecouta	12°36′46″ N 12°8′10″ W	B145	*E. gambianus*	M	0.24405196
-Jun-2021	Samecouta	12°36′46″ N 12°8′10″ W	B147	*E. gambianus*	M	0.14080196
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B178	*E. gambianus*	M	0.11717099
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B210	*E. gambianus*	F	0.55750196
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B215	*E. helvum*	M	0.15015196
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B216	*E. gambianus*	M	0.18795196
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B217	*E. gambianus*	M	0.41480196
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B219	*E. gambianus*	F	0.28045196
5-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B221	*M. pusillus*	M	0.03655196
7-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B227	*E. helvum*	M	0.24095196
7-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B230	*E. helvum*	M	0.12135196
7-Sep-2021	Ngari	12°38′5″ N 12°15′1″ W	B231	*E. helvum*	M	0.43235196
8-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B245	*E. gambianus*	M	0.47295196
8-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B260	*E. gambianus*	M	0.15515196
8-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B262	*E. gambianus*	M	0.36100196
8-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B264	*E. gambianus*	M	0.11325196
8-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B276	*E. gambianus*	M	0.07125196
8-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B289	*E. gambianus*	M	0.08770196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B295	*E. gambianus*	M	0.12675196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B296	*E. gambianus*	M	0.51830196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B304	*E. gambianus*	M	0.03450196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B311	*E. gambianus*	M	0.08945196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B313	*E. gambianus*	M	0.22530196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B314	*E. gambianus*	M	0.48400196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B316	*E. gambianus*	M	0.05605196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B322	*E. gambianus*	M	0.05400196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B327	*E. gambianus*	M	0.21035196
9-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B334	*E. gambianus*	M	0.10150196
10-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B341	*E. gambianus*	M	0.36105196
10-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B342	*E. gambianus*	M	0.00780196
10-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B344	*E. gambianus*	M	0.26105196
10-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B349	*E. gambianus*	M	0.04095196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B353	*E. gambianus*	M	0.36125196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B356	*E. gambianus*	M	0.96925196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B357	*E. gambianus*	F	0.12720196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B361	*E. gambianus*	M	0.55580196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B363	*E. gambianus*	M	0.38250196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B369	*E. gambianus*	F	0.37915196

Date	Location	GPS coordinates	ID	Species	Sex	CHIKV OD mean
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B370	*E. gambianus*	M	0.31105196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B372	*E. gambianus*	M	0.08705196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B375	*E. gambianus*	M	0.73960196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B376	*E. helvum*	M	0.84650196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B377	*E. helvum*	M	0.15215196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B380	*E. gambianus*	M	0.29785196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B381	*E. gambianus*	M	0.25020196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B387	*E. helvum*	M	0.19420196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B388	*E. helvum*	M	0.91235196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B389	*E. helvum*	M	1.32655196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B394	*E. gambianus*	F	0.34235196
11-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B396	*E. gambianus*	M	0.09005196
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B402	*E. gambianus*	M	0.17495196
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B403	*E. gambianus*	M	0.03795196
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B404	*E. gambianus*	M	0.09385196
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B407	*E. gambianus*	M	0.31339727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B409	*E. gambianus*	M	0.23734727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B414	*M. pusillus*	F	0.26889727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B416	*E. gambianus*	F	1.50979727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B418	*E. gambianus*	M	0.82364727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B422	*E. gambianus*	M	0.18199727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B423	*E. gambianus*	F	0.45509727
12-Sep-2021	Samecouta	12°36′46″ N 12°8′10″ W	B424	*E. gambianus*	M	0.11659727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B437	*E. gambianus*	M	0.10889727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B444	*E. gambianus*	M	1.22039727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B445	*M. pusillus*	M	0.24119727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B450	*M. pusillus*	M	0.55914727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B454	*E. helvum*	F	0.29589727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B455	*M. pusillus*	F	0.52494727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B457	*M. pusillus*	M	0.14374727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B458	*E. gambianus*	M	0.63804727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B459	*E. gambianus*	F	0.04049727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B465	*E. helvum*	F	0.49589727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B467	*E. helvum*	F	0.22682099
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B469	*E. helvum*	F	0.07179727
13-Sep-2021	Ndebou	12°30′21.08″ N 12°27′33.48″ W	B482	*E. helvum*	F	0.40562099

CHIKV, Chikungunya virus; F, female; GPS, Global Positioning System; ID, identification sample; M, male; na, not available; OD, optical density.



Appendix Figure. Measured optical density at 490 nm (OD490) in bat samples positive for antibodies against the envelope 2 recombinant protein of chikungunya virus using in-house ELISA protocol. Four bat species are presented: *Epomophorus gambianus* (n=63), *Eidolon helvum* (n=15), *Micropteropus pusillus* (n=7), *Epodomus franqueti* (n=3). The positive samples from *Lissonycteris angolensis* (n=1, with OD490=0.213) are not presented. The box plot shows the range of data from the median (middle line) to the 25th and 75th percentile (box boundaries), with the whisker lines showing the minimum and maximum values. Individual data points (i.e., OD490 mean) are shown as dots. No statistically significant differences were observed between groups using the unpaired t-test.