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

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

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

2. Materials and Methods

2. 1 Virus strains

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

2. 2 Cells lines

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

2. 3 Suckling mice

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

2. 4 Mosquitoes

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

2.5 Viral stock preparation

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

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

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

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

2.6 Growth kinetics

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

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

2.7 Oral infection of mosquitoes

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

2.8 Analysis of mosquito samples

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

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

Samples were considered positive when they were detected by RT-PCR and confirmed by IFA. The rates of infection (number of positive bodies/ number of tested mosquitoes), dissemination (number of positive legs-wings/ number of positive bodies) and transmission (number of positive

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

2.9 RNA extraction and real time RT-PCR

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

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

Both primers and probes systems were synthesised (TIB Mol-Biol, Berlin, Germany) and tested.

The real-time PCR assays were performed using the Quantitect Probe RT-PCR Kit (Qiagen, Heiden, Germany) in a 96-well plate under the following conditions: 50°C for 15 min, 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Copy numbers of genome were calculated using Ct (Cycle threshold) and corresponding RNA standard.

2.10 Development of USUVsub RT-PCR assay

2.10.1 Standard RNA for USUVsub

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

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

2.10.2 Determination of analytical specificity and sensitivity

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

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

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

2.11 Secondary structure analysis

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

3. Results

3.1 Viral stocks

For *in vitro* kinetic experiments, the viral stocks were prepared in C6/36 cells and the following titers were observed: $4.25x10^7$pfu/ml, $2x10^4$ pfu/ml and $3x10^5$pfu/ml, respectively for SAAR1776, HB81P08 and ArB1803.

For mosquito infections, higher viral titers were needed and the viral stocks were prepared in suckling mice. The following higher titers were observed: $3.5x10^{10}$pfu/ml, $3x10^{10}$pfu/ml and $1.35x10^9$pfu/ml, respectively for SAAR1776, HB81P08 and ArB1803.

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

3.2 Validation of USUV subtype RT-PCR assay

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

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

3.3 Growth kinetics

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

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

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

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

3.4 Vector competence of *Cx. quinquefasciatus* mosquitoes

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

One experimental infection was done for strain SAAR1776, and the blood meal titer post feeding (pfe) was $5x10^6$ pfu/ml. RT-PCRs tests detected infection rates of 40, 50, 60 and 44.61% for 4, 8,

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

For USUVsub (ArB1803), two experimental infections were done; the blood meal contained $5.75x10^7$ and $3.25x10^6$ pfu/ml. RT-PCRs tests detected infection rates between 40 to 50% at 4, 8 and 12 dpi for experiment 2. Infection rates were 19.51% and 38.70% at 15 dpi for experiments 1 and 2 respectively (Figure 5, columns A and B). However, IFA tests confirmed only 5 % of infection at 4 dpi and 20% at 8 and 12 dpi. For 15 dpi, 15.85% and 29.03% were confirmed respectively for both infections (Figure 5, upper panels columns A and B). Dissemination was shown for this strain by RT-PCR with rates of 30 and 44.44% at 4 and 8 dpi. The virus was not detected in the mosquito's legs-wings at 12 dpi. Dissemination rates were 58.33% and 18.75% at 15dpi for experiments 1 and 2 respectively (Figure 5, middle panels columns A and B). IFA tests confirmed dissemination only at 15 dpi with rates of 7.69% and 55.55% for both experiments (Figure 5). Transmission rates estimated by RT-PCR tests were 42.85% and 33.33% at 15 dpi for both experiments (Figure 5, lower panels columns A and B). The corresponding viral genome copy numbers in these positive salivas were determined using the RNA standard developed in this study and ranged between 1042 to 3232 per ml. However, no infectious particles were detected in the saliva samples by IFA and therefore the transmission of the USUVsub was not confirmed.

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

4. Discussion

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

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

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

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.

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

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

In Senegal, Nikolay *and colleagues* [Nikolay et al., 2012] showed that the mosquito *Cx. neavei* was able to transmit the USUV reference strain using a blood meal titer, which did not exceed $4.5x10^6$ pfu/ml. In our study we infected *Cx. quinquefasciatus* mosquitoes with the same strain with $5x10^6$ pfu/ml and for the others strains even higher viral titers (HB81P08: $4.5x10^7$pfu/ml, ArB1803: $3.25x10^6$ to $5.75x10^7$ pfu/ml) were used. In Europe, to investigate vector competence of *Cx. pipiens*, belonging to the same complex as *Cx. quinquefasciatus*, Fros *and colleagues* performed their oral infection with 50% tissue culture infectious dose ($TCID_{50}$) of $4x10^7$ per ml [Fros et al., 2015] and for *Aedes albopictus* $0.66x10^{7.5}$ / $0.66x10^{7.9}$ $TCID_{50}$/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.

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

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

5. Conclusions

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

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

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References

Aberle S.W., Kolodziejek J., Jungbauer C., Stiasny K., Aberle J.H., Zoufaly A., Hourfar M.K., Weidner L., Nowotny N. Increase in human West Nile and Usutu virus infections, Austria, 2018. Euro Surveill. 2018;23(43):pii=1800545.

Allering L, Jöst H, Emmerich P, Günther S, Lattwein E, Schmidt M, et al. Detection of Usutu virus infection in a healthy blood donor from south-west Germany, 2012. Euro Surveill. 2012;17:20341.

Bakonyi T, Jungbauer C, Aberle SW, Kolodziejek J, Dimmel K, Stiasny K, Allerberger F, Nowotny N. Usutu virus infections among blood donors, Austria, July and August 2017 - Raising awareness for diagnostic challenges. Euro Surveill. 2017 Oct;22(41).

Brugger K., Rubel F. Simulation of climate-change scenarios to explain Usutu-virus dynamics in Austria. *Prev Vet Med.* 2009; 88:24–31.

Busani L., Capelli G., Cecchinato M., Lorenzetto M., Savini G., Terregino C., Vio P., Bonfanti L., Pozza M.D., Marangon S. West Nile virus circulation in Veneto region in 2008-2009. *Epidemiol Infec*t. 2011; 139(6):818-25.

Cadar D., Becker N., Campos Rde M., Börstler J., Jöst H., Schmidt-Chanasit J. Usutu virus in bats, Germany, 2013. Emerg Infect Dis. 2014; 20(10):1771-3.

Cadar D., Maier P., Müller S., Kress J., Chudy M., Bialonski A., Schlaphof A., Jansen S., Jöst H., Tannich E., Runkel S., Hitzler W.E., Hutschenreuter G., Wessiepe M., Schmidt-Chanasit J. Blood donor screening for West Nile virus (WNV) revealed acute Usutu virus (USUV) infection, Germany, September 2016. Euro Surveill. 2017; 22(14).

Carletti F, Colavita F, Rovida F, Percivalle E, Baldanti F, Ricci I, et al. Expanding Usutu virus circulation in Italy: detection in the Lazio region, central Italy, 2017 to 2018. Euro Surveill. 2019;24:1800649.

Cavrini F., Gaibani P., Longo G., Pierro A.M., Rossini G., Bonilauri P., Gerunda G.E., Di Benedetto F., Pasetto A., Girardis M., Dottori M., Landini M.P., Sambri V. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. Euro Surveill. 2009; 14(50).

Cavrini F., Pepa M.E., Gaibani P., Pierro A.M., et *al.* A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. *J Clin Virol*. 2011; 50(3):221-3.

Chvala-Mannsberger S., Bakonyi T., Brugger K., Nowotny N., Weissenbock H. Epizootiology of Usutu virus associated bird mortality in Austria. Austrian Contributions to Veterinary Epidemiology. Vienna: Institute for Veterinary Public Health. 2007 Volume 4.

Danecek P., Schein C.H. Flavitrack analysis of the structure and function of West Nile non-structural proteins. *Int J Bioinform Res Appl.* 2010; 6(2):134-46.

De Madrid A.T., Porterfield J.S. A simple micro-culture method for the study of group B arboviruses. *Bull World Health Organ.* 1969; *40:* 113—121.

Diagne M.M, Ndione M.H.D., Di Paola N., Fall G., Bedekelabou A.P., Sembène P.M., Faye O., Zanotto P.M.A., Sall A.A. Usutu Virus Isolated from Rodents in Senegal. Viruses. 2019; 11(2). pii: E181.

Digoutte J.P., Calvo-Wilson M.A., Mondo M., Traore-Lamizana M., Adam F. Continuous cell lines and immune ascitic fluid pools in arbovirus detection. *Res Virol.* 1992; *143(6):* 417--422.

Domanović D., Gossner C.M., Lieshout-Krikke R., Mayr W., Baroti-Toth K., Dobrota A.M., Escoval M.A., Henseler O., Jungbauer C., Liumbruno G., Oyonarte S., Politis C., Sandid I., Vidović M.S., Young J.J., Ushiro-Lumb I., Nowotny N. West Nile and Usutu Virus Infections and Challenges to Blood Safety in the European Union. Emerging Infectious Diseases, 2019, Vol. 25.

Escribano-Romero E., Lupulovic´ D. Merino-Ramos T., Bla´zquez A., Lazic´ G., Lazic´ S., Saiz J., Petrovic´ T. West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. Veterinary Microbiology 2015; 176 365–369.

Fall G., Diallo M., Loucoubar C., Faye O., and Sall A. A. Vector Competence of *Culex neavei and Culex quinquefasciatus* (*Diptera: Culicidae*) from Senegal for Lineages 1, 2, Koutango and a Putative New Lineage of West Nile virus. Am. J. Trop. Med. Hyg., 2014; 90(4), pp. 747–754.

Fall G., Faye M., Weidmann M, Kaiser M., Dupressoir A., Ndiaye E.H., Ba Y., Diallo M., Faye O., Sall A.A. Real-Time RT-PCR Assays for Detection and Genotyping of West Nile Virus Lineages Circulating in Africa. Vector Borne Zoonotic Dis. 2016; 16(12):781-789.

Fall G., Di Paola N., Faye M., Dia M., Freire C.Cd.M., Loucoubar C., et al. Biological and phylogenetic characteristics of West African lineages of West Nile virus. PLoS Negl Trop Dis. 2017; 11 (11).

Fros J.J, Miesen P., Vogels C.B., Gaibani P., Sambri V., Martina B.E., Koenraadt C.J., van Rij R.P., Vlak J.M., Takken W., Pijlman G.P. Comparative Usutu and West Nile virus transmission potential by local *Culex pipiens* mosquitoes in north-western Europe. 2015; 1:31-36.

Gaibani P., Pierro A., Alicino R., Rossini G., Cavrini F., Landini M.P., Sambri V. Detection of Usutu-virus-specific IgG in blood donors from northern Italy. Vector Borne Zoonotic Dis. 2012; 12(5):431-3.

510 Gerberg E.J., Barnard D.R., Ward R.A. Manual for Mosquito Rearing and Experimental
511 Techniques. Am Mosq Control Assoc Bull. 1994; 61–62.
512 Gowda N.N., Vijayan V.A. Indoor resting density, survival rate and host preference of *Culex*
513 *quinquefasciatus* say (Diptera: *Culicidae*) in Mysore City. *J Commun Dis*.1992; *24(1):* 20--28.
514 Grottola A., Marcacci M., Tagliazucchi S., Gennari W., Di Gennaro A., Orsini M., Monaco
515 F., Marchegiano P., Marini V., Meacci M., Rumpianesi F., Lorusso A., Pecorari M., Savini G.
516 Usutu virus infections in humans: a retrospective analysis in the municipality of Modena, Italy.
517 Clin Microbiol Infect. 2017; 23(1):33-37.
518 Kerpedjiev P, Hammer S, Hofacker IL. Forna (force-directed RNA): Simple and effective online
519 RNA secondary structure diagrams. Bioinformatics. 2015 Oct 15;31(20):3377-9.
520 Lelli R., Savini G., Teodori L., Filipponi G., Di Gennaro A., Leone A., et *al*. Serological
521 evidence of USUTU virus occurrence in north-eastern Italy. *Zoonoses Public Health*. 2008;
522 55(7):361-7.
523 McIntosh B.M. Usutu (SA Ar 1776), nouvel arbovirus du groupe B. *Int Catalogue*
524 *Arboviruses*.1985; 3:1059–1060.
525 Moser LA, Boylan BT, Moreira FR, Myers LJ, Svenson EL, Fedorova NB, Pickett BE, Bernard
526 KA. Growth and adaptation of Zika virus in mammalian and mosquito cells. PLoS Negl Trop
527 Dis. 2018 Nov 12;12(11):e0006880.
528 Ndiaye E. H., Fall G., Gaye A., Bob N.S., Talla C., Diagne C.T., Diallo D., BA Y., Dia I., Kohl
529 A., Sall A. A. and Diallo M. Vector competence of *Aedes vexans (Meigen*), *Culex poicilipes*
530 *(Theobald) and Cx. quinquefasciatus Say* from Senegal for West and East African lineages of
531 Rift Valley fever virus. Parasites & Vectors. 2016; 9:94.

Nikolay B., Diallo M., BoyeC.S.b., and Sall A.A. Usutu Virus in Africa. Vector-Borne and Zoonotic Diseases, 2011.

Nikolay B., Diallo M., Faye O., Boye C. S., and Sall A. A. Vector Competence of *Culex neavei (Diptera: Culicidae)* for Usutu Virus. *Am J Trop Med Hyg.* 2012; 86(6):993-996.

Nikolay B., Dupressoir A., Firth C., Faye O., Boye C.S., Diallo M. and Sall A.A. Comparative full length genome sequence analysis of Usutu virus isolates from Africa, *Virology*. 2013a; 10:217.

Nikolay B., Weidmann M., Dupressoir A., Faye O., Boye C.S., Diallo M., Sall A.A. Development of a Usutu virus specific real-time reverse transcription PCR assay based on sequenced strains from Africa and Europe, *JofVirol Methods*. 2013b; 10.1016.

Ochieng C., Lutomiah J., Makio A., Koka H., Chepkorir E., Yalwala S., Mutisya J., Musila L., Khamadi S., Richardson J., Bast J., Schnabel D., Wurapa E. and Sang R. Mosquito-borne arbovirus surveillance at selected sites in diverse ecological zones of Kenya; 2007 – 2012, *Virology*. 2013; 10:140.

Patel P., Landt O., Kaiser M., Faye O., Koppe T., Lass U., Sall A. A. and Niedrig M.. Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. Virology Journal. 2013; 10:58.

Pecorari M. , Longo G., Gennari W., Grottola A., Sabbatini A.M.T., Tagliazucchi S., Savini G., Monaco F., Simone M.L., Lelli R., Rumpianesi F. First human case of usutu virus neuroinvasive infection, Italy, August-September 2009. *Eurosurveillance*. 2009; 14(50): pii=19446.

Percivalle E., Sassera D., Rovida F., Isernia P., Fabbi M., Baldanti F., Marone P. Usutu Virus Antibodies in Blood Donors and Healthy Forestry Workers in the Lombardy Region, Northern Italy. Vector Borne Zoonotic Dis. 2017; 17(9):658-661.

Poidinger M., Hall R.A., Mackenzie J.S. Molecular characterization of the Japanese encephalitis serocomplex of the flavivirus genus. *Virology*. 1996; 218:417–421.

Puggioli A., Bonilauri P., Calzolari M., Lelli D., Carrieri M., Urbanelli S., Pudar D., Bellini R. Does Aedes albopictus (Diptera: Culicidae) play any role in Usutu virus transmission in Northern Italy? Experimental oral infection and field evidences. Acta Trop. 2017; 172:192-196.

Santini M., Vilibic-Cavlek T., Barsic B., Barbic L., Savic V., Stevanovic V., Listes E., Di Gennaro A., Savini G. First cases of human Usutu virus neuroinvasive infection in Croatia, August-September 2013: clinical and laboratory features. J Neurovirol. 2015; 21(1):92-7.

Slonchak A, Khromykh AA. Subgenomic flaviviral RNAs: What do we know after the first decade of research? Antiviral Res. 2018 Nov;159:13-25.

Steinmetz H.W., Bakonyi T., Weissenböck H., Hatt J.M., Eulenberger U., Robert N. , Hoop R., Nowotny N. Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland—Genomic and pathologic comparison to other central European outbreaks. *Veterinary microbiology*. 2011; 148, (2–4): 207–212.

Simonin Y., Sillam O., Carles M.J., Gutierrez S., Gil P., Constant O., Martin M.F., Girard G., Van de Perre P., Salinas S., Leparc-Goffart I., Foulongne V. Human Usutu Virus Infection with Atypical Neurologic Presentation, Montpellier, France, 2016. Emerg Infect Dis. 2018; 24(5):875-878.

Stock N.K., Laraway H., Faye O., Diallo M., Niedrig M. and A. A. Sall. Biological and Phylogenetic Characteristics of Yellow Fever Virus Lineages from West Africa. *Virology*. 2013; 87(5):2895-2907.

Villordo SM, Filomatori CV, Sánchez-Vargas I, Blair CD, Gamarnik AV. Dengue virus RNA structure specialization facilitates host adaptation. PLoS Pathog. 2015 Jan 30;11(1):e1004604.

Weidmann M., Sall A.A., Manuguerra J.C., Koivogui L., et al. Quantitative analysis of particles, genomes and infectious particles in supernatants of haemorrhagic fever virus cell cultures. Virol J. 2011; 8:81.

Weissenböck H., Kolodziejek J., Url A., Lussy H., Rebel-Bauder B., Nowotny N. Emergence of *Usutu virus*, an African Mosquito-Borne *Flavivirus* of the Japanese Encephalitis Virus Group, Central Europe. *Emerg Infect Dis*. 2002; 8(7): 652–656.

Weissenböck H., Kolodziejek J., Fragner K., Kuhn R., Pfeffer M., Nowotny N. Usutu virus activity in Austria, 2001-2002. Microbes Infect. 2003; 5(12):1132-6.

Weissenböck H., Bakonyi T., Rossi G., Mani P., Nowotny N. Usutu virus, Italy, 1996. Emerg Infect Dis. 2013; 19(2):274-7.

Woodall J.P. The viruses isolated from arthropods at the East African Virus Research Institute in the 26 years ending December 1963. *Proc E Afc Acad.* 1964; II: 141-146.

Yeh SC, Pompon J. Flaviviruses Produce a Subgenomic Flaviviral RNA That Enhances Mosquito Transmission. DNA Cell Biol. 2018 Mar;37(3):154-159.

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	*Culex neavei*	7	AP3/NBM3/C61	AY453412
ArB1803	Central African Republic	1969	*Culex perfuscus*	7	AP3/NBM3/C61	KC754958
HB81P08	Central African Republic	1981	Human	7	AP3/NBM3/C61	KC754955

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′- CGTAGATGTTTTCAGCCCACGT	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-AAGAGAGAAGACATTTGGTGCGGCAGT—TMR	NS5
1803 NS5 F1 (USUVsub)	5′- CCGAGGACAGGATGAACTCA	NS5
1803 NS5 R1 (USUVsub)	5′- TGGCCTGACATTCCTACACT	NS5

Table 3: Specificity of the USUV subtype RT-PCR

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

Strains	Virus	Geographic origin	Host origin	Year of isolation	Panflavi primers	USUV Subtype primers
ArB1803	USUVsub	Central African Republic	*Culex perfuscus*	1969	31.55	21.74
SAAR1776	USUV	South Africa	*Culex neavei*	1959	26.17	-
HB81P08	USUV	Central African Republic	Human	1981	25.52	-
ArD101291	USUV	Senegal	*Culex gr. univittatus*	1993	24.67	-
259524	USUV	Senegal	*Mastomys natalensis*	2013	25.79	-
259520	USUV	Senegal	*Mastomys natalensis*	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	*Aedes vexans*	2002	37.41	-
Dak ArB209	Bagaza	Central African Republic	*Culex spp.*	1966	23.74	-
ArB490	Bouboui	Central African Republic	*Anopheles paludis*	1967	23.48	-
ArD14701	Kedougou	Senegal	*Aedes minutis*	1972	29.06	-
H177	Wesselsbron	South Africa	Human	1955	19.28	-

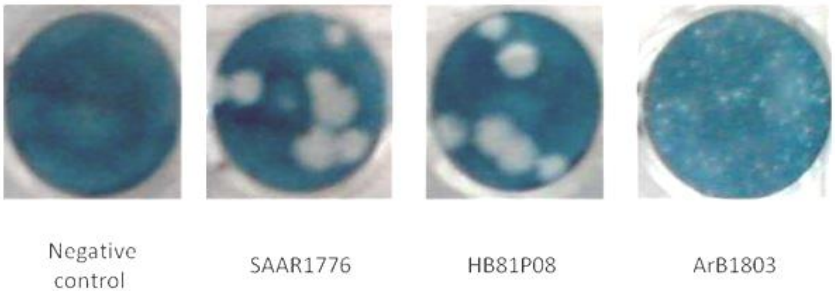
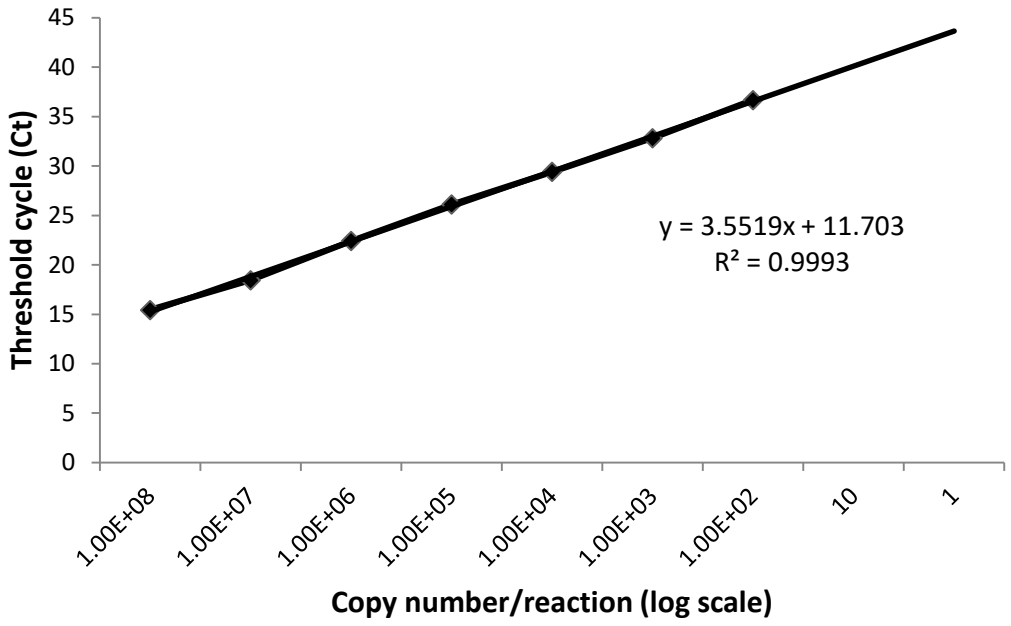
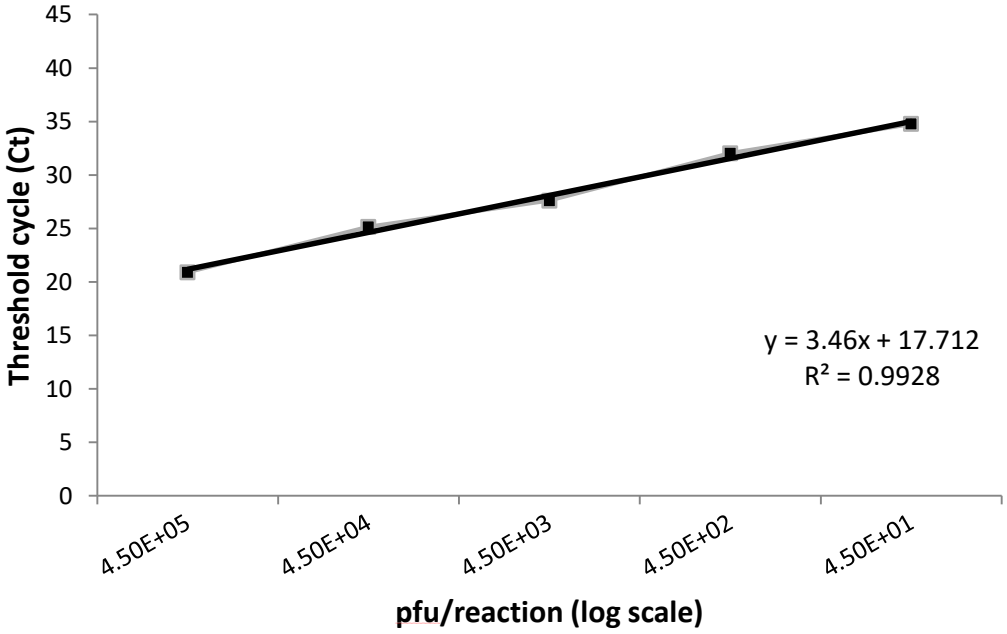


Figure 1: Titration of USUV strains. Shows the plaques obtained during USUV titration with PS cells.

685 **A**



686 **B**



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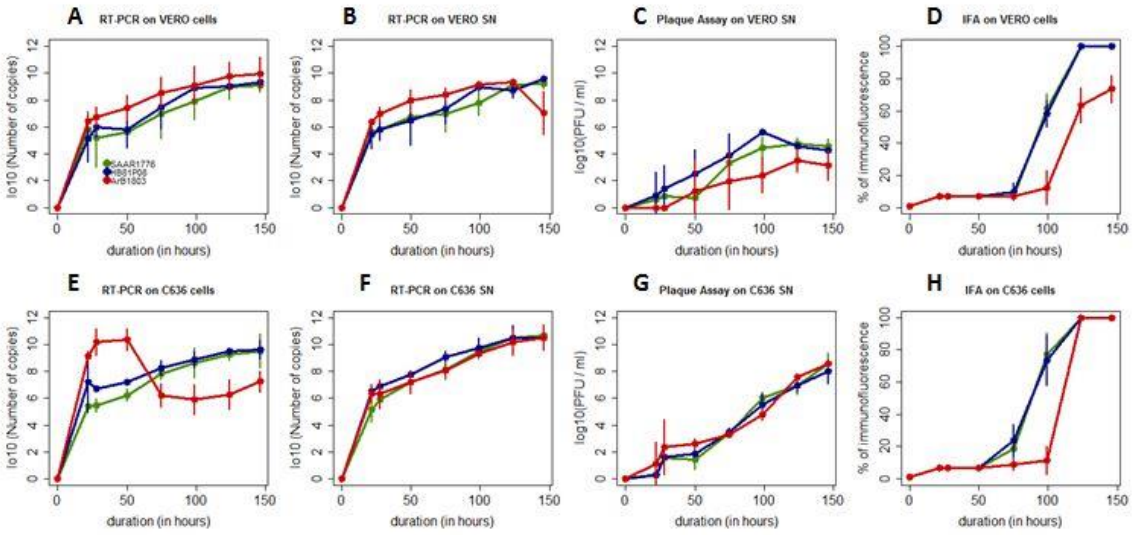
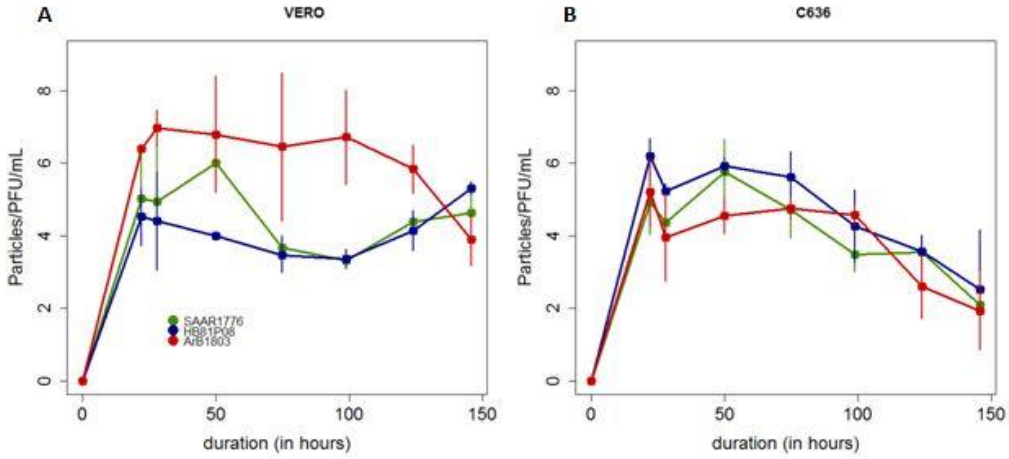
689 **<u>Figure 2</u>: Sensitivity of the USUV subtype RT-PCR assay**. **(A):** Serial 10-fold dilutions of *in*
690 *vitro* RNA standard have been tested in the corresponding real-time RT-PCR assay. Tested
691 dilutions ranged from 1×10^8 to 1 copies/reaction for the RNA standard of the USUVsub specific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

for USUVsub real-time RT-PCR assay. Tested dilutions ranged from $4.5x10^5$ to 4.5 pfu for the USUVsub viral stock.



<u>Figure 3</u>: Growth kinetics of different strains of USUV mammalian (VERO) and in mosquitoe (C6/36) cells. Amount of viral RNA equivalents isolated from cells (**A** and **E**) and from supernatant (**B** and **F**) (log10 of RNA copy number), the number of infectious viral particles (**C** and **G**) (log10 PFU/ml), and percentage of immunofluorescence of cells infected (**D** and **H**), and at 22, 28, 50, 75, 99, 124 and 146 hours pi. The experiments were performed with C6/36 cells (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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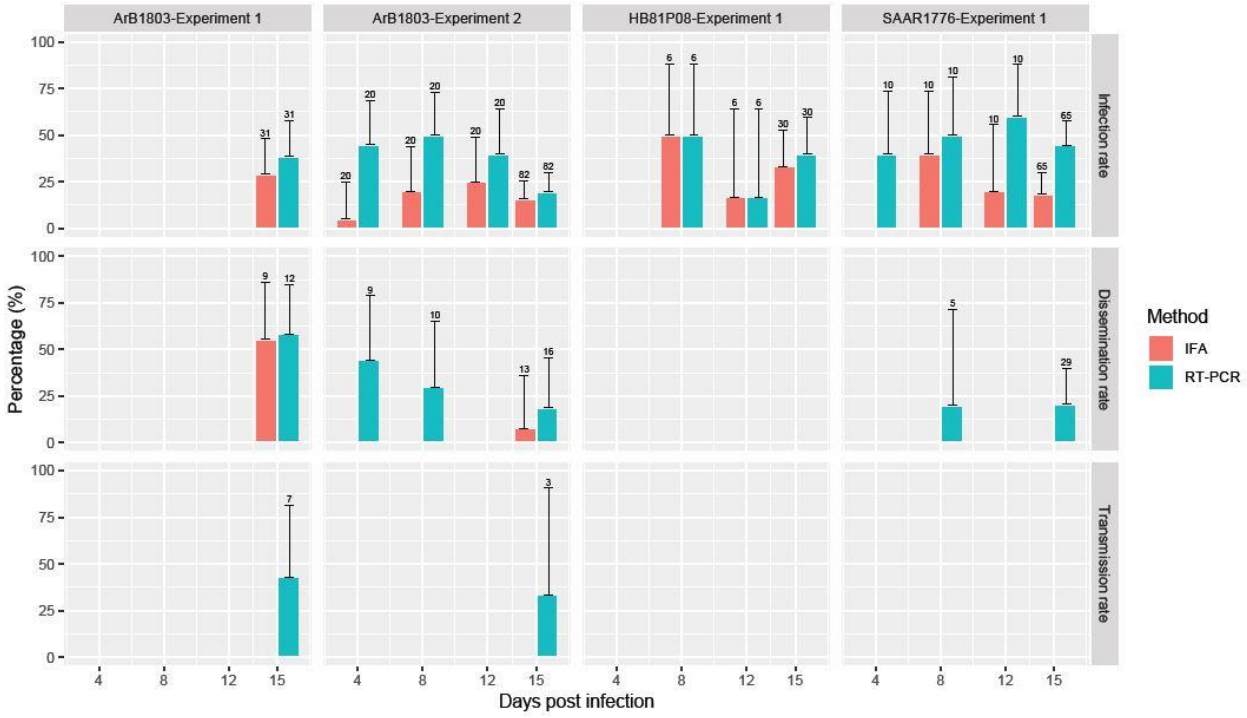

Figure 5: qRT-PCR and Indirect Immunofluorescence Assay (IFA) of bodies, legs-wings and saliva of mosquitoes infected with different strains of USUV.

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