Veterinary Microbiology 263 (2021) 109262

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

The envelope protein of Usutu virus attenuates West Nile virus virulence in immunocompetent mice

Guendalina Zaccaria [a], Daniela Malatesta [a], Lucija Jurisic [a,b], Maurilia Marcacci [a,c], Giovanni Di Teodoro [a], Annamaria Conte [a], Liana Teodori [a], Federica Monaco [a], Valeria Marini [a], Claudia Casaccia [a], Giovanni Savini [a], Annapia Di Gennaro [a], Emanuela Rossi [a], Vincenzo D'Innocenzo [a], Nicola D'Alterio [a], Alessio Lorusso [a,*]

[a] Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise (IZSAM), Campo Boario, 64100 Teramo, Italy
[b] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy
[c] Dipartimento di Medicina Veterinaria, University of Bari, Valenzano, Bari, Italy

ARTICLE INFO

Keywords:
Chimeric WNV/USUV
Mice
Reverse genetics
Genome manipulation

ABSTRACT

West Nile virus (WNV) and Usutu virus (USUV) are the two most widespread mosquito-borne flaviviruses in Europe causing severe neuroinvasive disease in humans. Here, following standardization of the murine model with wild type (wt) viruses, we engineered WNV and USUV genome by reverse genetics. A recombinant virus carrying the 5′ UTR of WNV within the USUV genome backbone (r-USUV$_{5'\text{-UTR WNV}}$) was rescued; when administered to mice this virus did not cause signs or disease as wt USUV suggesting that 5′ UTR of a marked neurotropic parental WNV was not *per se* a virulence factor. Interestingly, a chimeric virus carrying the envelope (E) protein of USUV in the WNV genome backbone (r-WNV$_{E\text{-USUV}}$) showed an attenuated profile in mice compared to wt WNV but significantly more virulent than wt USUV. Moreover, except when tested against serum samples originating from a live WNV infection, r-WNV$_{E\text{-USUV}}$ showed an identical antigenic profile to wt USUV confirming that E is also the major immunodominant protein of USUV.

1. Introduction

Flaviviruses (genus *Flavivirus*, family *Flaviviridae*) are enveloped, positive-sense single-stranded RNA viruses of about 11,000 nucleotides (nt) (Lindenbach et al., 2007). The flavivirus genome is translated as a single open reading frame flanked by 5′ (which contains a 5′-cap structure) and 3′ untranslated regions (UTR) but lacking a polyA tail at the 3′end (Ng et al., 2017). In general, the polyprotein is cleaved by host and viral proteases into three structural proteins, the capsid (C), pre-membrane/membrane (prM/M) and envelope (E), which contribute to the viral structural elements, as well as seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Roby et al., 2015) which regulate viral replication. According to the International Committee on Taxonomy of Viruses (2020), viruses belonging to the *Flavivirus* genus are organized in 53 viral species (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/360/genus-flavivirus). The majority of flaviviruses that are relevant to human and animal diseases are organized into serocomplexes (Calisher et al., 1989). Serocomplexes are defined by the ability of polyclonal post-immune sera against one flavivirus of the same serocomplex to neutralize others (St. John and Rathore, 2019). Indeed, interpretation of serological results can be challenging, principally due to the extensive cross-antigenic reactivity between the members of the *Flavivirus* genus (Musso and Despres, 2020). Serological characteristics of flaviviruses mainly depend on the E protein. The E protein facilitates membrane fusion between the virus and host cell (Hu et al., 2021) and is the major target for neutralizing antibodies inducing protective immunity (Heinz and Stiasny, 2012). Neutralization tests are now recognized as gold standard for differentiating flaviviral infections on a serological basis (Musso and Despres, 2020).

West Nile virus (WNV) belongs to the Japanese encephalitis virus (JEV) serogroup and is transmitted by infected *Aedes* and *Culex* mosquitoes (Colpitts et al., 2012). WNV is maintained in an enzootic cycle between mosquitoes and birds but can also infect and cause disease in horses and humans, which serve as incidental dead-end hosts (McLean et al., 2002; Kramer et al., 2007; Savini et al., 2013; Llopis et al., 2015;

* Corresponding author.
E-mail address: a.lorusso@izs.it (A. Lorusso).

https://doi.org/10.1016/j.vetmic.2021.109262
Received 14 August 2020; Accepted 17 October 2021
Available online 21 October 2021
0378-1135/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Mancini et al., 2017). Humans may develop a mild flu-like illness consisting of symptoms such as malaise, eye pain, headache, myalgia, gastrointestinal distress, and rash. However, 1 out of 150 infected persons will show neurological signs of infection which may also develop into a neuroinvasive disease with a manifestation of meningitis, encephalitis, and acute flaccid paralysis (Campbell et al., 2002; Petersen and Marfin, 2002; Debiasi and Tyler, 2006). Immunocompromised patients, the elderly, children and people with underlying conditions are especially at risk of developing severe disease (Sejvar and Marfin, 2006; Kramer et al., 2007; Sejvar, 2014). WNV circulates in the field in viral lineages (L) with L1 and L2 as the most prevalent in Europe (Bakonyi et al., 2006). Usutu virus (USUV) also belongs to JEV serogroup (Weissenböck et al., 2004; Lorusso et al., 2019) and it has been classified in several putative African and European lineages (Cadar et al., 2015; Engel et al., 2016). USUV natural life cycle is such as that of WNV. Mammals including humans, horses or wild boars are described as accidental or dead-end hosts (Nikolay et al., 2011; Barbic et al., 2013; Escribano-Romero et al., 2015). Since 2009, some neurological disorders such as encephalitis, meningitis and meningoencephalitis were found associated with USUV-infection in immunocompromised and immunocompetent patients (Grottola et al., 2017; Salinas et al., 2017).

Susceptibility of immunocompetent adult mice to USUV is limited (Blázquez et al., 2013), whereas mice lacking the interferon type 1 receptor are susceptible, as described for other flaviviruses such as ZIKA virus (Dowall et al., 2016; Lazear et al., 2016; Martín-Acebes et al., 2016; Tripathi et al., 2017). Quite the opposite, WNV has been characterized extensively in the murine model (reviewed in (Graham et al., 2017)). Within this milieu, since the WNV emergence in New York-USA in 1999 (Lanciotti et al., 1999), intensive work has been performed on the critical viral proteins and host factors implicated in WNV virulence and immune-pathogenesis. With the exception of the C and NS2B sequence regions, molecular determinants for mammalians have been identified in all parts of the WNV genome (reviewed by Fiacre et al., 2020). Overall, the E glycoprotein is the major determinant for WNV virulence in mice and the glycosylation site within the E glycoprotein may modulate its pathogenicity.

In this study, following standardization of the murine model with several parental viruses, we engineered WNV and USUV genome by reverse genetics and demonstrated that the introduction of the E protein of USUV in the WNV genome backbone attenuates WNV virulence in mice and that the 5′ UTR of a marked neutropic WNV strain is not a virulence factor when introduced within USUV genome.

2. Materials and methods

2.1. Ethics

All procedures on animals were accomplished respecting the European and Italian regulations on the use of animals for experimental purposes. All efforts were made to minimize animal suffering. The study was approved by the Animal Welfare Committee of IZSAM and authorized by the Italian Ministry of Health (authorization number 48/2015-PR and 472/2019-PR).

2.2. Cells and viruses

BSR cells (CCLV-RIE-582, Institute Pasteur, Paris-France) and African green monkey kidney cells (Vero, ATCC CRL-1586) were maintained at 37 °C in humified atmosphere with 5 % CO_2 in a minimal essential medium (MEM; Biowest) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U penicillin, 100 µg/mL streptomycin). Two wild type (wt) WNV L1 strains (wt WNV L1 15803/2008 (acc. no. FJ483579), and wt WNV L1 20652/2012 (acc. no. MW835364)), one wt WNV L2 strain (wt WNV L2 20168/2012 (acc. no. MW862073)), one wt USUV strain (wt USUV 12543/2010 (acc. no. KX555624), Europe 2 lineage), and three rescued chimeric viruses (recombinant wild-type (r-wt) WNV, r-$WNV_{E\text{-}USUV}$, and r-$WNV_{5'\text{-}UTR\ WNV}$) were employed for animal experiments. Wild type and rescued viruses were propagated onto Vero cells maintained in MEM supplemented with 3 % heat inactivated FBS and antibiotics. Viral stocks used for animal work were sequenced before infecting animals. Viral stock for r-wt WNV was obtained at passage 2 on cell culture whereas for the remaining viruses, either parental or chimeric, viral stocks were obtained at the fourth cell passage. Amino acid (aa) divergence in the E protein was absent between wt WNV L1 15803/2008 and wt WNV L1 20652/2012. They both share the 95.4 % of aa identity with wt WNV L2 20168/2012. USUV E protein shares the 78 % of aa identity with L1 strains and the 77 % with the L2 strain.

2.3. Animals

Specific pathogen-free (SPF), male and female 6–8 weeks-old Swiss-CD1 mice were purchased from a commercial vendor (Charles River Laboratories, Inc) and used in three independent *in vivo* experiments. Mice were housed in a vector-free animal facility, under controlled environmental conditions with free access to food and water with a 12 -h day-night cycle. Animals unable to move or too disoriented to reach food or water were euthanized by cervical dislocation as well as mice surviving to the studies. Blood was collected by puncture of the submandibular vein in a tube without anticoagulant.

2.4. Necropsy and sampling

As for *in vivo* studies, immediately after death or euthanasia, fresh samples of spleen, liver, intestine, kidney, hearth, lung, spinal cord, whole brain and eye were collected and immediately processed as described below. Part of the tissues was also immersed in 10 % buffered formalin for histological and immunohistochemical investigations.

2.5. Histopathology and immunohistochemistry

Collected tissues were fixed in 10 % formalin and embedded in paraffin-wax. 5µm thick sections were examined using haematoxylin and eosin (HE) staining and visualized by light microscopy. Serial tissues sections were dewaxed, rehydrated, and processed for immunohistochemistry (IHC). IHC was performed with a streptavidin-biotin peroxidase complex method, using a specific antibody anti-WNV M glycoprotein (final dilution: 1:500, rabbit polyclonal antibody, Abcam, ab22070). Sections were incubated in 3 % hydrogen peroxide in absolute methanol for 30 min to inhibit endogenous peroxidase activity then rinsed in 0.05 M Tris-buffered saline (TBS), pH 7.6, for 5 min. Antigen retrieval was performed by heat treatment in citrate buffer 0.01 M pH 6.0 at 121 °C for 5 min. To reduce nonspecific binding, slides were incubated in 20 % normal goat serum (Vector Laboratories, Inc., USA) in TBS for 30 min, followed by overnight incubation with primary antibody in a humidified chamber at 4 °C. The day after, slides were rinsed in TBS before a second incubation for 30 min with biotinylated goat anti-rabbit antibody (final dilution: 1:200, Vector Laboratories, Inc., USA). Immune reactions were carried out by means of an ABC complex (Vectastain Elite, Vector Laboratories, Inc., USA). Antibody binding was visualized with 3−3'- diaminobenzidine solution (Dako, Glostrup, Denmark), applied for 1 min, followed by a light counterstain with Mayer's haematoxylin (Bio-Optica, Milan, Italy). Positive and negative controls were included in each IHC run. The specificity of the immunolabeling was verified with an irrelevant antibody directed against an unrelated antigen.

2.6. Molecular assays

Tissues, except for the brain, were homogenized 1:5 in phosphate-buffered saline with antibiotics. Nucleic acids were purified by means of the BioSprint 96 One-For-All Vet kit, Qiagen. To quantify viral burden

in the brain, half the brain was weighed and homogenized using a metal bead in 1 mL of sterile MEM containing antibiotics (100 U penicillin, 100 µg/mL streptomycin).

All tissues were tested by a quantitative real-time reverse transcription (RT) polymerase chain reaction (PCR) for WNV ($qPCR_{WNV}$, (Eiden et al., 2010)) and USUV ($qPCR_{USUV}$, (Cavrini et al., 2011)) according to the experimental groups. $qPCR_{WNV}$ targets a 64 bp fragment of the genome region which codes for the NS2A protein of both WNV lineages, whereas $qPCR_{USUV}$ targets a 73 bp fragment of the genome region coding for the NS5. RNA copy numbers for both viruses were quantified using a standard curve of *in vitro* transcribed RNA of known quantities (data not shown). The limit of detection with 95 % probability, established by Probit analysis, was 5 and 7 RNA copies/µl for $qPCR_{WNV}$ and $qPCR_{USUV}$, respectively (data not shown).

2.7. Reverse genetics strategy and plasmids

We used the infectious subgenomic amplicons (ISA, (Aubry et al., 2014)) method for rescuing engineered viruses. Plasmids containing the virus sequences (*de novo* synthesis by GenScript, Hong Kong, China) were constructed as described (Aubry et al., 2014) to generate WNV and USUV and served as template for double-stranded DNA fragments production. The human cytomegalovirus promoter (pCMV) sequence and the hepatitis delta ribozyme sequence followed by the simian virus 40 polyadenylation signal (HDR/SV40pA) sequence were inserted at the 5′ terminus of the first DNA fragment and at the 3′ terminus of the last DNA fragment, respectively. The following viral strains were selected for plasmid synthesis: strain WNV L1 15803/2008 (acc. no. FJ483549) and strain USUV_Italia_2009 (acc. no. JF266698, Europe 2 lineage). Scrutiny, wt USUV 12543/2010 and strain USUV_Italia_2009 differ for 7 amino acid (aa) in the polyprotein precursor (total length 3434 aa), one of which is located in the E protein (S595G). Importantly, strain USUV_Italia_2009 has the same biological features in mice of wt USUV 12543/2010 (data not shown) and selected for genome manipulation because it was assumed as the Italian USUV reference strain. Moreover, USUV_Italia_2009 was one of the few Italian USUV strains, at the time plasmids were designed, with complete 5′ and 3′ UTRs publicly available and both belonged to the same Europe 2 lineage of USUV. Nevertheless, before cloning, USUV_Italia_2009 has been sequenced again following procedures described above. The obtained sequence was identical to that of the deposited sequence.

2.8. Chimeric plasmid production

A plasmid harboring the USUV E protein coding sequence, in the WNV genome backbone, was constructed. This was achieved by means of the one-step DNA fragment assembly and circularization system described previously (Zuo and Rabie, 2010). Briefly, the WNV DNA fragment containing the E protein coding sequence, obtained from PCR amplification (PfuUltra High-Fidelity DNA Polymerase kit, Agilent; 4.3×10^{-7} errors per nt per cycle) of the Genscript WNV plasmid (pCC1-WNV_fr1), was cloned into the pGEM-T Easy Vector (Promega, pGEM-WNV_fr2). The linear vector backbone of WNV was produced by PCR amplification (PfuUltra High-Fidelity DNA Polymerase kit, Agilent) of pGEM-WNV_fr2 while USUV E protein coding sequence employing the first GenScript USUV plasmid as template (pUC57-USUV_fr1). The whole process was based on the use of primers in both PCRs that included complementary directional overhangs of WNV linear vector backbone extremities to obtain two overlapping PCR products. Single-step DNA assembly and circularization was performed on non-purified PCR products, using the PfuUltra High-Fidelity DNA Polymerase kit (Agilent) following manufacturer's instructions, without adding any primers to the PCR mix. A plasmid harboring WNV 5′ UTR sequence in the USUV genome backbone was also constructed as described above. The two chimeric plasmids were verified by Sanger sequencing. Primer sequences are available upon request.

2.9. Preparation of DNA fragments

The complete genome flanked by the pCMV and HDR/ SV40pA sequences was amplified by PCR in three overlapping DNA fragments for WNV, USUV and for the USUV chimeric virus harboring WNV 5′ UTR sequence (r-$USUV_{5'\text{-}U}TRWNV$). A strategy based on four overlapping DNA fragments was used for the WNV chimeric virus harboring USUV E protein coding sequence (r-$WNV_{E\text{-}USUV}$) using plasmids as template (primer sequences are available upon request). Amplicons were produced using the PfuUltra High-Fidelity DNA Polymerase kit (Agilent) following manufacturer's instructions. PCR products were verified by gel electrophoresis and purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions, and then DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermofisher Scientific).

2.10. Cell transfection

The transfection protocol was adapted from (Aubry et al., 2014) and (Atieh et al., 2017). A final amount of 3 µg of an equimolar mixture of sub-genomic amplicons was incubated with 12 µl of Lipofectamine 2000 (Invitrogen) in 250 µl of Opti-MEM medium (Life Technologies), following manufacturer's instructions. The mixture was added to a 6 wells culture plate of 80 % confluent BSR cells containing 1 mL of MEM without antibiotics. As negative control one well was transfected with an equimolar mixture of only two subgenomic amplicons. As positive control of the transfection efficiency, one well was transfected with the pEGFP-C1 vector containing the eGFP gene under the control of the pCMV. After 4 h of incubation the cell supernatant was removed, cells were washed twice with MEM and 2 mL of fresh medium (MEM with 3% heat inactivated FBS) were added. The cell supernatant was harvested when cytopathic effect (CPE) was observed or 7 days post-transfection if CPE was not evident, centrifuged and stored at −80 °C. Each virus was then passaged using Vero cells and the cell supernatant was harvested when CPE was observed. Three blind passages in Vero cells were conducted when CPE was not observed. Clarified cell supernatants were used to perform direct immunofluorescence assay, $TCID_{50}$ assay, and whole genome sequencing by NextSeq 500 platform (Illumina).

2.11. Direct immune-fluorescence assay (dIFA)

Direct IFA was performed using 4-well cell culture chamber slides of Vero cells infected using the clarified cell supernatant originating from cell transfections. When CPE was observed, supernatant was removed, and cells washed twice with PBS. The slides were then dried, plunged 20 min in cold acetone for fixation, dried again and incubated 30 min at 37 °C with appropriately diluted polyclonal anti-M glycoprotein WNV antibodies (final dilution: 1:200, ab22070, Abcam), which can detect WNV and USUV. After incubation, slides were washed twice with PBS, dried, incubated 30 min at 37 °C with appropriately diluted FITC-conjugated secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI), washed twice with PBS, washed once with distilled water, dried, and observed using a fluorescence microscope.

2.12. Genome sequencing

Before inoculation in mice, parental and rescued viruses were sequenced. Whole genome sequencing of nucleic acids purified from the 2nd passage of r-wt WNV and from the 4th passage of the extant viruses, was performed by NGS (Marcacci et al., 2016). Sequences were analyzed using the SeqMan module of DNASTAR software (DNASTAR, Inc. Madison, WI, USA). Bioinformatics analysis was performed as described previously by our group (Marcacci et al., 2016) by mapping against reference sequences. 5′ UTR of USUV 12543/2010, r-$USUV_{5'\text{-}U}TRWNV$, and USUV_Italia_2009 were obtained using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen), following

manufacturer's instructions. Amplicon sequencing was performed in an ABI3130XL sequencer using ABI PRISM Big Dye® Terminator (Applied Biosystems).

2.13. Serum neutralization assay

Wild type viruses and r-WNV$_{E\text{-}USUV}$ were tested by serum neutralization (SN) using anti-WNV and anti-USUV serum samples. These serum samples were obtained from laboratory animals either infected (mice, data not shown) or immunized (rabbits, (Lorusso et al., 2019)) with live or inactivated, respectively, WNV and USUV strains. SN assay was performed following procedures described previously in detail by our group (Di Gennaro et al., 2014). The antibody titer was defined as the reciprocal of the highest dilution of the serum that showed 100 % neutralization. Positive and negative control sera were included in each plate. Sera with titer of 10 were considered positive.

2.14. In vivo studies

Three *in vivo* studies were performed. In each study, animals were monitored daily for 21 days. The first and the second *in vivo* studies were assessed to investigate the lethality and kinetics of parental wt viruses in mice, respectively. Details are available upon request.

The last *in vivo* study (experiment 3) was instead planned to investigate lethality and kinetics of engineered viruses. A total of 60 mice were employed for this experiment. Three independent groups of 15 mice were administered intraperitoneally (IP) with 150 µL 10^4 $TCID_{50}$/mL (titrated by $TCID_{50}$) of the fourth cell passage of each virus obtained by ISA, including r-wt WNV, r-WNV$_{E\text{-}USUV}$ and r-USUV$_{5'\text{-}UTR\ WNV}$. Three additional groups of 5 mice each were added and administered with 150 µL of sterilized MEM, wt WNV and wt USUV, respectively. Only animals found dead or who were euthanized following the development of severe clinical signs were necropsied and sampled. From survivors, a serum sample was collected before being sacrificed and tested by serum-neutralization against wt WNV, wt USUV, r-wt USUV$_{5'\text{-}UTR\ WNV}$ and r-WNV$_{E\text{-}USUV}$ following procedures described below.

2.15. Data analysis

All comparisons among groups were analyzed by the Kruskal-Wallis nonparametric test. P-values equal to or less than 0.05 were statistically significant. Multiple pairwise comparisons were explored through the Dunn *post hoc* method and significance values have been adjusted by the Bonferroni correction for multiple tests. The analyses were performed in R software version 3.3.2 (R Core team, https://www.r-project.org/) and visualized in GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Strain, lineage or dose do not affect WNV virulence in mice and wild type WNVs showed strong tropism for the central nervous system

Briefly, no differences in clinical signs and lethality (100 %) were observed regardless of WNV lineage, strain, or dose. USUV-infected mice did not show any clinical sign and all of them survived the experiment. As expected, the highest WNV RNA titers were detected in the central nervous system (CNS); at 6 dpi, regardless of the WNV strain, mean titers in the CNS ranged between 10^7 and 10^8 RNA copies per gram of brain tissue. No differences in terms of type of histological lesions were observed. Detailed results are available upon request.

3.2. r-wt WNV and two chimeric WNV/USUV were rescued by ISA

r-wt WNV was successful rescued from transfected BSR cells whereas, despite several attempts, r-wt USUV was not rescued. Thus, we decided to replace each portion of USUV genome with homologous regions of WNV and the first replacement involved the 5′ UTR. Two chimeric viruses including r-WNV$_{E\text{-}USUV}$ and r-USUV$_{5'\text{-}UTR\ WNV}$ (Fig. 1 A-top) were successfully rescued.

Virus replication of chimeric viruses was demonstrated by the evidence of CPE and the detection of viral antigens by dIFA. Instead, r-wt USUV was not rescued (Fig. 1 A-bottom). Chimeric viruses showed the desired genome constellation. No differences in genome sequences were evidenced in chimeric rescued viruses used for animal studies with respect to the donor parental viruses. 5′ UTR sequence (a total of 96 bp) of USUV 12543/2010 showed 5-point mutations in the first 14 nucleotides in comparison with USUV_Italia_2009. Nevertheless, both types of 5′ UTR are present in USUV sequences available in GenBank.

3.3. r-WNV$_{E\text{-}USUV}$ showed a lower lethality rate and a weaker CNS tropism than wt WNV; r-USUV$_{5'\text{-}UTR\ WNV}$ has the same in vivo features of wt USUV

Significant differences in survival were observed between r-USUV$_{5'\text{-}UTR\ WNV}$ and r-WNV$_{E\text{-}USUV}$ ($P = 0.003$), between wt WNV and r-WNV$_{E\text{-}USUV}$ ($P < 0.0001$), and between r-wt WNV and r-WNV$_{E\text{-}USUV}$ ($P < 0.0001$). No differences were observed between wt WNV and r-wt WNV ($P = 0.2853$). Mortality in mice infected with wt USUV, r-USUV$_{5'\text{-}UTR\ WNV}$ (Fig 1B, purple line) and sterile MEM was not observed. All wt WNV-administered mice died within 8 dpi (Fig. 1B, green line). Nearly all mice infected with r-wt WNV died within 10 dpi, except for one individual which was euthanized at the end of the study (Fig. 1B, red line). r-WNV$_{E\text{-}USUV}$ was responsible for the death of 46 % (7/15) inoculated mice. The first died at 9 dpi, the last at 14 dpi (Fig. 1B, blue line). When present, clinical signs induced by r-wt WNV and r-WNV$_{E\text{-}USUV}$ groups were not different from those observed in wt WNV-inoculated mice. r-USUV$_{5'\text{-}UTR\ WNV}$ and wt USUV-administered mice did not show any clinical signs and all of them survived the experiment. Lower RNA titers were observed in the CNS of r-WNV$_{E\text{-}USUV}$-inoculated mice with respect to those observed in wt and r-wt WNV inoculated mice (Fig. 1C). RNA titers in the brain tissue showed an overall significant difference among groups ($K\text{–}W = 14.5$ $p < 0.01$), with *a post hoc* analysis revealing a difference between r-wt WNV and r-WNV$_{E\text{-}USUV}$ ($p = 0.000$). The difference between wt WNV and r-WNV$_{E\text{-}USUV}$ was not statistically significant (although remarkably close as for $p = 0.018$ with a Bonferroni correction significance $p = 0.0167$), likely due to the small sample size of wt WNV ($n = 5$). Results for the other tissues are available upon request. An overall significant difference among the three groups was evidenced, with *a post hoc* analysis revealing a difference between r-wt WNV and r-WNV$_{E\text{-}USUV}$ and between wt WNV and r-WNV$_{E\text{-}USUV}$.

Interestingly, only a minimal increase of microglial cells and scattered mild neuronal degenerations were observed in mice succumbed by r-WNV$_{E\text{-}USUV}$ at 9 and 10 dpi (3/7 mice) (Fig. 2A). Lesions were not evidenced in other organs as well as in the individual succumbed at 14 dpi (1/7 mice). Three mice of these WNV challenged mice succumbed at 9 dpi were autolytic and not suitable for histopathological investigations. Viral antigens were also detected in neurons of brain (Fig. 2B), spinal cord and myenteric ganglia (Fig. 2C).

At the end of the experiment, a blood sample was collected from survivors. WNV antibody titer of 80 was observed in the survivor administered with r-wt WNV. All wt USUV, r-USUV$_{5'\text{-}UTR}$WNV and r-WNV$_{E\text{-}USUV}$- administered mice showed scarce or no neutralizing antibody response for USUV, irrespective of the strain, either parental or chimeric, used. Results are available upon request.

3.4. The E is the major immunodominant protein of Usutu virus

Parental WNV, USUV and r-WNV$_{E\text{-}USUV}$ were tested by SN against WNV and USUV positive serum samples. These serum samples originated from mice and rabbits infected or immunized with live and inactivated (Lorusso et al., 2019) WNV and USUV viruses. Results are

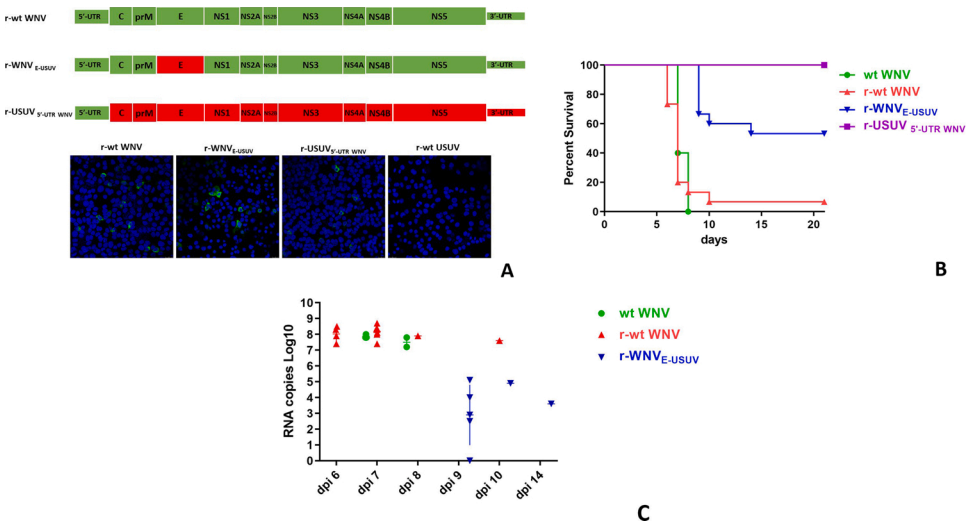

Fig. 1. A-top. Genomic constellation of recombinant (r) wt WNV and chimeric recombinant viruses rescued by reverse genetics. Green boxes, WNV genome; red boxes, USUV genome. For reverse genetics experiments, only wt WNV L1 15803 was used and therefore referred to as wt WNV. Analogously, JF266698 of strain USUV_Italia_2009 would have been the genetic source of r-wt USUV and its recombinant thereof. However, r-wt USUV was not rescued. **A-bottom**. Direct IFA using 4-well cell culture chamber slides of Vero cells infected using the clarified cell supernatant originating from cell transfections. The slides were then dried, plunged 20 min in cold acetone for fixation, dried again and incubated 30 min at 37 °C with appropriately diluted polyclonal anti-M glycoprotein WNV antibodies (ab22070, Abcam), which can detect WNV and USUV. Green, FITC-conjugated secondary antibody; blue, DAPI. **B**. Survival curves of mice inoculated intraperitoneally (ip) with 150 µL 10^4 $TCID_{50}$/mL of r-wt WNV (N = 15), r-$WNV_{E\text{-}USUV}$ (n = 15), r-$USUV_{5'\text{-}UTR\text{-}WNV}$ (n = 15), wt WNV (n = 5), wt USUV (n = 5) and sterile MEM (n = 5). Significant differences in survival were observed between r-$USUV_{5'\text{-}UTR\text{-}WNV}$ and r-$WNV_{E\text{-}USUV}$ (P = 0.003), between wt WNV and r-wt WNV both with r-$WNV_{E\text{-}USUV}$ (P < 0.001). No differences were observed between wt WNV and r-wt WNV (P = 0.2853). Mortality in groups of mice infected with wt USUV, r-$USUV_{5'\text{-}UTR\ WNV}$, and sterile MEM was not observed. Sterile MEM and wt USUV-infected mice were not included in the graph. Animals succumbed following viral infection were sampled and analyzed as described previously. Serum samples were collected from survived animals and then euthanized (21 dpi). **C.** Viral burden in the brains of mice infected with 150 µL 10^4 $TCID_{50}$/mL of r-wt WNV (N = 15), r-$WNV_{E\text{-}USUV}$ (n = 15), r-$USUV_{5'\text{-}UTR\ WNV}$ (n = 15), wt WNV (n = 5), wt USUV (n = 5) and sterile MEM (n = 5). Viral burden was measured in terms of RNA copies (mean ± SD) per gram of brain. Nearly all mice infected with r-wt WNV died within 10 dpi, except for one individual which was euthanized at the end of the study (red line). All wt WNV administered mice died within 8 dpi (green line). r-$WNV_{E\text{-}USUV}$ was responsible for the death of 46 % (7/15) inoculated mice. The first mice died at 9 dpi, the last at 14 dpi (purple line). Three of these WNV challenged mice succumbed at 9 dpi but were autolytic and not suitable for histopathological investigations. $USUV_{5'\text{-}UTR\ WNV,}$ wt USUV and sterile MEM-administered mice did not show any clinical signs and all of them survived to the experiment. Viral burdens in the other tissues are available upon request.

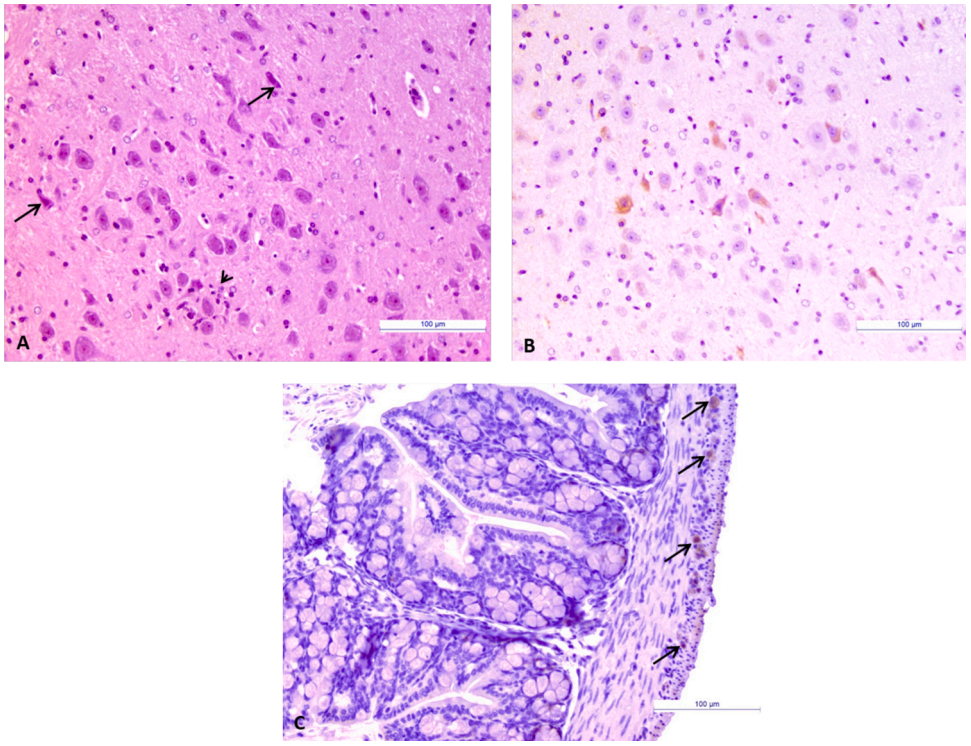

Fig. 2. CNS, brainstem of mice succumbed following r-$WNV_{E\text{-}USUV}$ (A); few degenerated and shrunken neurons (arrows) with multifocal glial cells (arrowhead); 20X, (EE). (B) CNS, brainstem, WNV antigens (brown) in few neurons and microglial cells, 20X, (IHC). (C) Gut, large intestine: WNV antigens in scattered neurons of myenteric ganglia (arrows); 20X, (IHC).

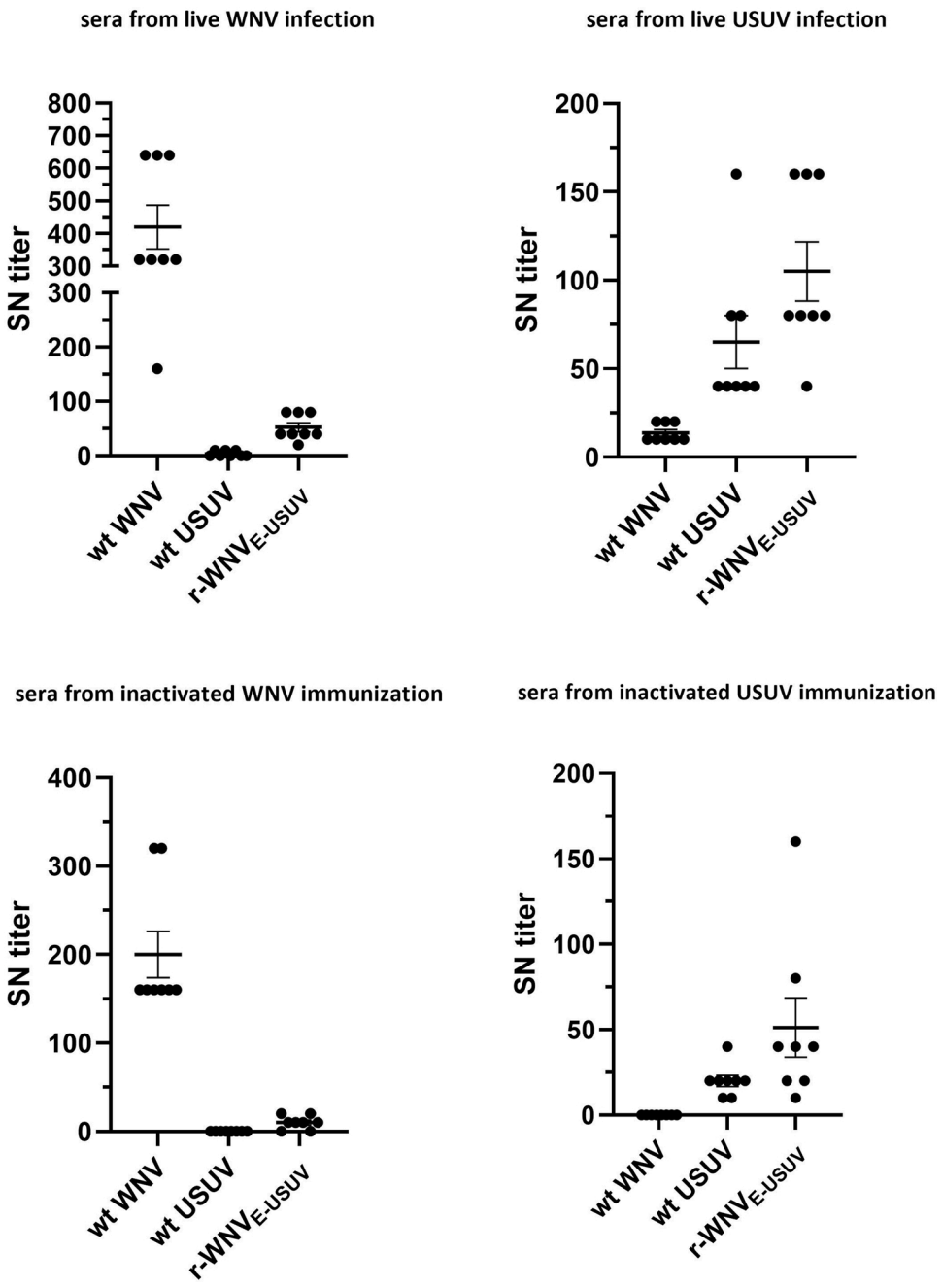

Fig. 3. Wild type WNV and USUV and r-WNV$_{E\text{-}USUV}$ were tested by serum neutralization (SN) using anti-WNV and anti-USUV serum samples. These samples were obtained from laboratory animals immunized with live (mice, data not shown) and inactivated (rabbits, (Lorusso et al., 2019)) WNV and USUV strains. SN assay was performed following procedures described previously in detail by our group (Lorusso et al., 2019). The antibody titer was defined as the reciprocal of the highest dilution of the serum that showed 100 % neutralization. Positive and negative control sera were included in each plate. Sera with titer of 10 were considered positive.

summarized in Fig. 3. Both types of USUV antisera neutralized wt USUV and r-WNV$_{E\text{-}USUV}$ in a similar manner; scarce cross reactivity with wt WNV was observed with antisera originating from a live USUV infection (Fig. 3, top right). Neutralization did not occur between WNV and sera mounted following immunization with inactivated USUV (Fig. 3, bottom right); sera originating from immunization with inactivated WNV (Lorusso et al., 2019) did not neutralize wt USUV and only scarce neutralization of few serum samples was observed with r-WNV$_{E\text{-}USUV}$ (Fig. 3, bottom left). Neutralization of r-WNV$_{E\text{-}USUV}$ was clearly evidenced when WNV antisera originating from a live infection were employed (Fig. 3, top left) as this chimeric virus retained, except for the E protein, the genome backbone of WNV.

4. Discussion

In this study we manipulated USUV genome by reverse genetics. The introduction of the USUV E protein encoding gene in the WNV backbone resulted in the rescue and replication of chimeric virus r-WNV$_{E\text{-}USUV}$. This virus exhibited intermediate *in vivo* characteristics between USUV and WNV. The E protein of USUV reduced the virulence of wt neurotropic WNV in terms of timing for onset of clinical signs, mortality, viral titers in the internal organs and lesions corroborating the evidence that the E protein is the major virulence determinant of WNV. The highest RNA titers of r-WNV$_{E\text{-}USUV}$ were demonstrated, as WNV, in the CNS. This single E protein switch was sufficient to decrease the virulence of WNV and, in the opposite scenario, the WNV backbone was able to permit the neuroinvasion of a virus carrying USUV E protein, a phenomenon that was not observed during *in vivo* infections with wt USUV and r-USUV$_{5'\text{-}UTR\ WNV}$.

The E protein facilitates membrane fusion between the virus and host cells being therefore responsible for entry of flaviviruses into cells,. and E glycosylation plays important roles in viral attachment and cell entry,

replication, transmission, and pathogenesis (reviewed in (Carbaugh and Lazear, 2020). All parental strains possessed a N-glycosylation site at position 154 of the E protein (NYS) which was apparently retained also in strains rescued by ISA. Most WNV strains, including virulent strains such as NY99 are glycosylated at N154, but some strains as Kunjin are not. A well-known study which compared WNV strains from the 1999 outbreak in New York (Nash et al., 2001) to historical WNV strains, demonstrated that E glycosylation was associated with increased brain infection and lethality in mice (Shirato et al., 2004). A following study confirmed these findings by generating infectious clones of glycosylated virulent (NY99) and non-glycosylated attenuated (ETH76a) WNV strains and measuring their lethality in mice (Beasley et al., 2005). In our experimental setting, wt USUV and r-USUV$_{5'\text{-UTR WNV}}$ were not detected in the CNS of infected mice as opposite as r-WNV$_{E\text{-USUV}}$; therefore, the presence of the glycosylation at N154 is not *per se* a virulence factor but rather a strain-specific virulence feature.

The chimeric r-USUV$_{5'\text{-UTR WNV}}$ was successfully rescued by ISA and did not cause any clinical disease in mice, thus resembling wt USUV. It has been reported that the 5′ UTR might play an essential role in the neuropathogenic properties of WNV strain NY99 (Audsley et al., 2011) but not demonstrated for other WNV strains (Hussmann et al., 2013; Alsaleh et al., 2016). Our study suggested that the 5′ UTR of WNV L1 15803/2008 was not a virulence factor as we did not observe increased USUV virulence in immune-competent mice. It remains unknown why we failed the rescue of r-wt USUV. Our failure is, indeed, in stark contrast with the recent rescue of two synthetic r-wt USUV obtained by reverse genetics starting from sequence information of two modern USUV strains (Bates et al., 2021). Nevertheless, our r-USUV$_{5'\text{-UTR WNV}}$ resembles wt-USUV and therefore could reasonably serve as USUV genomic backbone for future studies.

Our study has certainly few shortcomings. First, we did not rescue a chimeric r-WNV$_{E\text{-USUV}}$ deprived of the *N-linked* glycosylation site at position 154. This latter experiment would eventually better clarify the role of the E glycosylation in USUV neuroinvasion and neurovirulence in the context of a virulent genomic backbone. Second, inflammatory and cellular response in mice infected with parental and rescued viruses were not investigated. With certainty, analysis of these aspects may contribute to explain the differences observed in the clinical and histopathological outcomes. Moreover, E protein encoding genes from additional USUV lineages need to be cloned in similar experiments as it was recently demonstrated that, although all neurotropic, USUV strains belonging to the currently known lineages have different virulence profiles in six-day-old neonatal Swiss mice inoculated intraperitoneally (Clé et al., 2021).

In this study, cross reaction between WNV and USUV has been clearly evidenced when sera originating from live infections were used in neutralization tests. r-WNV$_{E\text{-USUV}}$ has been neutralized by immune sera raised following infection with live USUV similarly to wt USUV. Also, neutralization of r-WNV$_{E\text{-USUV}}$ by sera originating from a live WNV infection occurred but with a lesser extent if compared to wt WNV. This phenomenon could be mainly related to the presence of non-cross reactive anti WNV NS1 antibodies as confirmed by the absence of neutralization for wt USUV with the same set of serum samples (Kitai et al., 2011; Cleton et al., 2017). The similar neutralization profile of r-WNV$_{E\text{-USUV}}$ and wt USUV evidenced with the two USUV antisera types clearly demonstrates that the rescued chimeric virus was antigenically more similar to wt USUV rather than WNV, and that, consequently, the E protein is, as expected, the major immunodominant protein of USUV.

5. Conclusions

In conclusion, in this study we demonstrated that when the USUV E protein is introduced into the WNV backbone, the chimeric virus r-WNV$_{E\text{-USUV}}$ showed moderate virulence with respect to WNV suggesting that the E protein of USUV has less virulent properties, in the used animal model, when compared to the homologous of WNV. At the opposite, when the 5′ UTR of WNV is introduced in the USUV backbone, the resulting chimeric virus was not pathogenic in mice suggesting that 5′ UTR was not a virulence factor in our experimental setting. Further efforts are currently ongoing to unravel the inflammatory and cellular response in r-WNV$_{E\text{-USUV}}$ and to clone the WNV E protein within the USUV backbone. This study certainly opens novel avenues to pathogenesis study involving USUV.

Declaration of Competing Interest

The authors declare that conflicts of interests do not exist.

Acknowledgements

Funding of IZSAM were provided by the Italian Ministry of Health (IZS AM 01/18 RC, Ricerca Corrente 2018 “Studio della interazione di due flavivirus nel modello murino e aviare”, recipient Alessio Lorusso and IZS AM 01/12 “Evoluzione genomica ed antigenica del virus della West Nile in Italia e studio dei fattori di virulenza”, recipient Giovanni Savini). Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the IZSAM.

References

Alsaleh, K., Khou, C., Frenkiel, M.P., Lecollinet, S., Vàzquez, A., de Arellano, E.R., Després, P., Pardigon, N., 2016. The E glycoprotein plays an essential role in the high pathogenicity of European-Mediterranean IS98 strain of West Nile virus. Virology 492, 53–65. https://doi.org/10.1016/j.virol.2016.02.009.

Atieh, T., Nougairède, A., Klitting, R., Aubry, F., Failloux, A.B., De Lamballerie, X., Priet, S., 2017. New reverse genetics and transfection methods to rescue arboviruses in mosquito cells. Sci. Rep. 7 https://doi.org/10.1038/s41598-017-14522-6.

Aubry, F., Nougairède, A., de Fabritus, L., Querat, G., Gould, E.A., de Lamballerie, X., 2014. Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. J. Gen. Virol. 95, 2462–2467. https://doi.org/10.1099/vir.0.068023-0.

Audsley, M., Edmonds, J., Liu, W., Mokhonov, V., Mokhonova, E., Melian, E.B., Prow, N., Hall, R.A., Khromykh, A.A., 2011. Virulence determinants between New York 99 and Kunjin strains of West Nile virus. Virology 414, 63–73. https://doi.org/10.1016/j.virol.2011.03.008.

Bakonyi, T., Ivanics, É., Erdélyi, K., Ursu, K., Ferenczi, E., Weissenböck, H., Nowotny, N., 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, Central Europe. Emerg. Infect. Dis. 12, 618–623. https://doi.org/10.3201/eid1204.051379.

Barbic, L., Vilibic-Cavlek, T., Listes, E., Stevanovic, V., Gjenero-Margan, I., Ljubin-Sternak, S., Pem-Novosel, I., Listes, I., Mlinaric-Galinovic, G., Di Gennaro, A., Savini, G., 2013. Demonstration of Usutu virus antibodies in horses, Croatia. Vector-Borne Zoonotic Dis. 13, 772–774. https://doi.org/10.1089/vbz.2012.1236.

Bates, T.A., Chuong, C., Hawks, S.A., Rai, P., Duggal, N.K., Weger-Lucarelli, J., 2021. Development and characterization of infectious clones of two strains of Usutu virus. Virology 554, 28–36. https://doi.org/10.1016/j.virol.2020.12.004.

Beasley, D.W., Whiteman, M.C., Zhang, S., Huang, C.Y., Schneider, B.S., Smith, D.R., Gromowski, G.D., Higgs, S., Kinney, R.M., Barrett, A.D., 2005. Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. J. Virol. 79 (13), 8339–8347. https://doi.org/10.1128/JVI.79.13.8339-8347.2005.

Blázquez, A.B., Escribano-Romero, E., Merino-Ramos, T., Saiz, J.C., Martín-Acebes, M.A., 2013. Infection with usutu virus induces an autophagic response in mammalian cells. PLoS Negl. Trop. Dis. 7 https://doi.org/10.1371/journal.pntd.0002509.

Cadar, D., Bosch, S., Jöst, H., Börstler, J., Garigliany, M.M., Becker, N., Schmidt-Chanasit, J., 2015. Putative lineage of novel African usutu virus, central Europe. Emerg. Infect. Dis. 21, 1647–1650. https://doi.org/10.3201/eid2109.142026.

Calisher, C.H., Karabatsos, N., Dalrymple, J.M., Shope, R.E., Porterfield, J.S., Westaway, E.G., Brandt, W.E., 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol. 70, 37–43. https://doi.org/10.1099/0022-1317-70-1-37.

Campbell, G.L., Marfin, A.A., Lanciotti, R.S., Gubler, D.J., 2002. West Nile virus. Lancet Infect. Dis. https://doi.org/10.1016/S1473-3099(02)00368-7.

Carbaugh, D.L., Lazear, H.M., 2020. Flavivirus envelope protein glycosylation: impacts on viral infection and pathogenesis. J. Virol. 94 https://doi.org/10.1128/jvi.00104-20.

Cavrini, F., Della Pepa, M.E., Gaibani, P., Pierro, A.M., Rossini, G., Landini, M.P., Sambri, V., 2011. A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. J. Clin. Virol. 50, 221–223. https://doi.org/10.1016/j.jcv.2010.11.008.

Clé, M., Constant, O., Barthelemy, J., Desmetz, C., Martin, M.F., Lapeyre, L., Cadar, D., Savini, G., Teodori, L., Monaco, F., Schmidt-Chanasit, J., Saiz, J.C., Gonzales, G., Lecollinet, S., Beck, C., Gosselet, F., Van de Perre, P., Foulongne, V., Salinas, S., Simonin, Y., 2021. Differential neurovirulence of Usutu virus lineages in mice and

neuronal cells. J. Neuroinflammation 18 (1), 11. https://doi.org/10.1186/s12974-020-02060-4.
Cleton, N.B., van Maanen, K., Bergervoet, S.A., Bon, N., Beck, C., Godeke, G.J., Lecollinet, S., Bowen, R., Lelli, D., Nowotny, N., Koopmans, M.P.G., Reusken, C.B.E. M., 2017. A serological protein microarray for detection of multiple cross-reactive flavivirus infections in horses for veterinary and public health surveillance. Transbound. Emerg. Dis. 64, 1801–1812. https://doi.org/10.1111/tbed.12569.
Colpitts, T.M., Conway, M.J., Montgomery, R.R., Fikrig, E., 2012. West Nile Virus: biology, transmission, and human infection. Clin. Microbiol. Rev. 25, 635–648. https://doi.org/10.1128/CMR.00045-12.
Debiasi, R.L., Tyler, K.L., 2006. West Nile virus meningoencephalitis. Nat. Clin. Pract. Neurol. 2, 264–275. https://doi.org/10.1038/ncpneuro0176.
Di Gennaro, A., Lorusso, A., Casaccia, C., Conte, A., Monaco, F., Savini, G., 2014. Serum neutralization assay can efficiently replace plaque reduction neutralization test for detection and quantitation of west nile virus antibodies in human and animal serum samples. Clin. Vaccine Immunol. 21, 1460–1462. https://doi.org/10.1128/CVI.00426-14.
Dowall, S.D., Graham, V.A., Rayner, E., Atkinson, B., Hall, G., Watson, R.J., Bosworth, A., Bonney, L.C., Kitchen, S., Hewson, R., 2016. A susceptible mouse model for zika virus infection. PLoS Negl. Trop. Dis. 10 https://doi.org/10.1371/journal.pntd.0004658.
Eiden, M., Vina-Rodriguez, A., Hoffmann, B., Ziegler, U., Groschup, M.H., 2010. Two new real-time quantitative reverse transcription polymerase chain reaction assays with unique target sites for the specific and sensitive detection of lineages 1 and 2 West Nile virus strains. J. Vet. Diagn. Invest. 22, 748–753. https://doi.org/10.1177/104063871002200515.
Engel, D., Jöst, H., Wink, M., Börstler, J., Bosch, S., Garigliany, M.M., Jöst, A., Czajka, C., Lühken, R., Ziegler, U., Groschup, M.H., Pfeffer, M., Becker, N., Cadar, D., Schmidt-Chanasit, J., 2016. Reconstruction of the evolutionary history and dispersal of Usutu virus, a neglected emerging arbovirus in Europe and Africa. MBio 7. https://doi.org/10.1128/mBio.01938-15.
Escribano-Romero, E., Lupulović, D., Merino-Ramos, T., Blázquez, A.B., Lazić, G., Lazić, S., Saiz, J.C., Petrović, T., 2015. West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. Vet. Microbiol. 176, 365–369. https://doi.org/10.1016/j.vetmic.2015.02.005.
Fiacre, L., Pagès, N., Albina, E., Richardson, J., Lecollinet, S., Gonzalez, G., 2020. Molecular determinants of West Nile virus virulence and pathogenesis in vertebrate and invertebrate hosts. Int. J. Mol. Sci. 21 (23), 9117. https://doi.org/10.3390/ijms21239117.
Graham, J.B., Swarts, J.L., Lund, J.M., 2017. A mouse model of west nile virus infection. Curr. Protoc. Mouse Biol. 7, 221–235. https://doi.org/10.1002/cpmo.33.
Grottola, A., Marcacci, M., Tagliazucchi, S., Gennari, W., Di Gennaro, A., Orsini, M., Monaco, F., Marchegiano, P., Marini, V., Meacci, M., Rumpianesi, F., Lorusso, A., Pecorari, M., Savini, G., 2017. Usutu virus infections in humans: a retrospective analysis in the municipality of Modena, Italy. Clin. Microbiol. Infect. 23, 33–37. https://doi.org/10.1016/j.cmi.2016.09.019.
Heinz, F.X., Stiasny, K., 2012. Flaviviruses and flavivirus vaccines. Vaccine 30, 4301–4306. https://doi.org/10.1016/j.vaccine.2011.09.114.
Hu, T., Wu, Z., Wu, S., Chen, S., Cheng, A., 2021. The key amino acids of E protein involved in early flavivirus infection: viral entry. Virol. J. 18, 136–148. https://doi.org/10.1186/s12985-021-01611-2.
Hussmann, K.L., Samuel, M.A., Kim, K.S., Diamond, M.S., Fredericksen, B.L., 2013. Differential replication of pathogenic and nonpathogenic strains of West Nile Virus within astrocytes. J. Virol. 87, 2814–2822. https://doi.org/10.1128/jvi.02577-12.
Kitai, Y., Kondo, T., Konishi, E., 2011. Non-structural protein 1 (NS1) antibody-based assays to differentiate West Nile (WN) virus from Japanese encephalitis virus infections in horses: effects of WN virus NS1 antibodies induced by inactivated WN vaccine. J. Virol. Methods 171, 123–128. https://doi.org/10.1016/j.jviromet.2010.10.012.
Kramer, L.D., Li, J., Shi, P.Y., 2007. West Nile virus. Lancet Neurol. https://doi.org/10.1016/S1474-4422(07)70030-3.
Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., et al., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286 (5448), 2333–2337. https://doi.org/10.1126/science.286.5448.2333.
Lazear, H.M., Govero, J., Smith, A.M., Platt, D.J., Fernandez, E., Miner, J.J., Diamond, M. S., 2016. A mouse model of Zika virus pathogenesis. Cell Host Microbe 19, 720–730. https://doi.org/10.1016/j.chom.2016.03.010.
Lindenbach, B.D., Thiel, H.-J., Rice, C.M., 2007. Flaviviridae: the viruses and their replication, p 1101–1152. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, fifth ed, vol 1. Lippincott, Williams & Wilkins, Philadelphia, PA (modificare in introduction e mettere Lindenbach et al., 2007, sono 3 autori e non due).
Llopis, I.V., Rossi, L., Di Gennaro, A., Mosca, A., Teodori, L., Tomassone, L., Grego, E., Monaco, F., Lorusso, A., Savini, G., 2015. Further circulation of West Nile and Usutu viruses in wild birds in Italy. Infect. Genet. Evol. 32, 292–297. https://doi.org/10.1016/j.meegid.2015.03.024.
Lorusso, A., Marini, V., Di Gennaro, A., Ronchi, G.F., Casaccia, C., Carelli, G., Passantino, G., D'Alterio, N., D'Innocenzo, V., Savini, G., Monaco, F., Horton, D.L., 2019. Antigenic relationship among zoonotic flaviviruses from Italy. Infect. Genet. Evol. 68, 91–97. https://doi.org/10.1016/j.meegid.2018.11.023.
Mancini, G., Montarsi, F., Calzolari, M., Capelli, G., Dottori, M., Ravagnan, S., Lelli, D., Chiari, M., Santilli, A., Quaglia, M., Federici, V., Monaco, F., Goffredo, M., Savini, G., 2017. Specie di zanzare coinvolte nella circolazione dei virus della West Nile e Usutu in Italia. Vet. Ital. 53, 97–110. https://doi.org/10.12834/VetIt.114.933.4764.2.
Marcacci, M., De Luca, E., Zaccaria, G., Di Tommaso, M., Mangone, I., Aste, G., Savini, G., Boari, A., Lorusso, A., 2016. Genome characterization of feline morbillivirus from Italy. J. Virol. Methods 234. https://doi.org/10.1016/j.jviromet.2016.05.002.
Martín-Acebes, M.A., Blázquez, A.B., Cañas-Arranz, R., Vázquez-Calvo, Á., Merino-Ramos, T., Escribano-Romero, E., Sobrino, F., Saiz, J.C., 2016. A recombinant DNA vaccine protects mice deficient in the alpha/beta interferon receptor against lethal challenge with Usutu virus. Vaccine 34, 2066–2073. https://doi.org/10.1016/j.vaccine.2016.03.015.
McLean, R.G., Ubico, S.R., Bourne, D., Komar, N., 2002. West Nile virus in livestock and wildlife. Curr. Top. Microbiol. Immunol. https://doi.org/10.1007/978-3-642-59403-8_14.
Musso, D., Despres, P., 2020. Serological diagnosis of flavivirus-associated human infections. Diagnostics 10, 302. https://doi.org/10.3390/diagnostics10050302, 2020.
Nash, D., Mostashari, F., Fine, A., Miller, J., O'Leary, D., Murray, K., Huang, A., Rosenberg, A., Greenberg, A., Sherman, M., Wong, S., Layton, M., 1999 West Nile Outbreak Response Working Group, 2001. The outbreak of West Nile virus infection in the New York City area in 1999. N. Engl. J. Med. 344 (24), 1807–1814. https://doi.org/10.1056/NEJM200106143442401.
Ng, W.C., Soto-Acosta, R., Bradrick, S.S., Garcia-Blanco, M.A., Ooi, E.E., 2017. The 5' and 3' untranslated regions of the flaviviral genome. Viruses. https://doi.org/10.3390/v9060137.
Nikolay, B., Diallo, M., Boye, C.S.B., Sall, A.A., 2011. Usutu virus in Africa. Vector-Borne Zoonotic Dis. https://doi.org/10.1089/vbz.2011.0631.
Petersen, L.R., Marfin, A.A., 2002. West Nile virus: a primer for the clinician. Ann. Intern. Med. https://doi.org/10.7326/0003-4819-137-3-200208060-00009.
Roby, J.A., Setoh, Y.X., Hall, R.A., Khromykh, A.A., 2015. Post-translational regulation and modifications of flavivirus structural proteins. J. Gen. Virol. https://doi.org/10.1099/vir.0.000097.
Salinas, S., Constant, O., Desmetz, C., Barthelemy, J., Lemaitre, J.M., Milhavet, O., Nagot, N., Foulongne, V., Perrin, F.E., Saiz, J.C., Lecollinet, S., Van de Perre, P., Simonin, Y., 2017. Deleterious effect of Usutu virus on human neural cells. PLoS Negl. Trop. Dis. 11 https://doi.org/10.1371/journal.pntd.0005913.
Savini, G., Puggioni, G., DI Gennaro, a, DI Francesco, G., Rocchigiani, a M., Polci, a, Marini, V., Pinoni, C., Rolesu, S., Marruchella, G., Lorusso, A., Monaco, F., 2013. West Nile virus lineage 2 in Sardinian wild birds in 2012: a further threat to public health. Epidemiol. Infect. 141, 2313–2316. https://doi.org/10.1017/S0950268812003147.
Sejvar, J.J., 2014. Clinical manifestations and outcomes of West Nile virus infection. Viruses. https://doi.org/10.3390/v6020606.
Sejvar, J.J., Marfin, A.A., 2006. Manifestations of West Nile neuroinvasive disease. Rev. Med. Virol. 16, 209–224. https://doi.org/10.1002/rmv.501.
Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H., Takashima, I., 2004. Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. J. Gen. Virol. 85 (Pt 12), 3637–3645. https://doi.org/10.1099/vir.0.80247-0.
St. John, A.L., Rathore, A.P.S., 2019. Adaptive immune responses to primary and secondary dengue virus infections. Nat. Rev. Immunol. https://doi.org/10.1038/s41577-019-0123-x.
Tripathi, S., Balasubramaniam, V.R.M.T., Brown, J.A., Mena, I., Grant, A., Bardina, S.V., Maringer, K., Schwarz, M.C., Maestre, A.M., Sourisseau, M., Albrecht, R.A., Krammer, F., Evans, M.J., Fernandez-Sesma, A., Lim, J.K., García-Sastre, A., 2017. A novel Zika virus mouse model reveals strain specific differences in virus pathogenesis and host inflammatory immune responses. PLoS Pathog. 13 https://doi.org/10.1371/journal.ppat.1006258.
Weissenböck, H., Bakonyi, T., Chvala, S., Nowotny, N., 2004. Experimental Usutu virus infection of suckling mice causes neuronal and glial cell apoptosis and demyelination. Acta Neuropathol. 108, 453–460. https://doi.org/10.1007/s00401-004-0916-1.
Zuo, P., Rabie, B.M., 2010. One-step DNA fragment assembly and circularization for gene cloning. Curr. Issues Mol. Biol. 12, 11–16 doi:10.217.